

B-Lymphocytes are Elevated in Mouse Bone Marrow by Estrogen Deficiency, and Induce Receptor Activator of Nuclear Factor κ B Ligand (RANKL) Expression in Osteoblasts *via* Cell Adhesion

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Loss of estrogen caused by ovariectomy (OVX) stimulates bone resorption and bone marrow B-lymphopoiesis, resulting in a marked bone loss and an accumulation of B cells in mouse bone marrow. In OVX mice, the expression of receptor activator of nuclear factor κ B ligand (RANKL) mRNA was elevated in trabecular bone and bone marrow compared with sham mice. To examine the roles of B-lymphocytes in bone resorption, 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. The expression of RANKL mRNA in osteoblasts was markedly elevated by the contact with the fixed B cells, and the induction rate of RANKL was correlated with the number of B cells added. Treatment with inhibitors of ERK 1/2 and p38 MAP kinases suppressed the B cell-induced RANKL expression in osteoblasts, suggesting the involvement of these kinases in the signals via the cell-to-cell contact. These findings emphasize the roles of B-lymphocytes in RANKL-induced osteoclastogenesis and in pathogenesis of bone loss due to estrogen deficiency.

Key words — bone marrow, B-lymphocyte, sex steroids, bone resorption, osteoporosis

INTRODUCTION

Osteoporosis is the most common bone disease. It is recognized that postmenopausal women lose bone due to a decrease in ovarian estrogen. Ovariectomized (OVX) animals have been used to understand the mechanism of bone loss due to estrogen deficiency. We have reported that loss of estrogen stimulates B-lymphopoiesis in OVX mice, resulting in an accumulation of B cells in mouse bone marrow.¹⁾ It was assumed that the increased B-lymphopoiesis caused by estrogen deficiency was involved in stimulated bone resorption. This is because the increased B-lymphopoiesis induced by the administration of interleukin-7 (IL-7), resulted in marked bone loss caused by stimulated osteoclastic bone resorption in mice with intact ovarian function.²⁾ On the other hand, previous studies suggested that bone-resorbing cytokines such as IL-1, IL-6 and tumor necrosis factor α may be involved in bone loss in OVX mice.³⁻⁵⁾ Therefore, the mechanism of bone loss in OVX mice is still controversial.

Recently, the mechanisms of bone resorption have been actively examined and the discovery of the receptor activator of NF- κ B ligand (RANKL) has allowed elucidation of the mechanism by which osteoclasts differentiate in bone.⁶⁻⁹⁾ Osteoblasts express RANKL as a membrane-associated factor in response to bone-resorbing factors such as 1α , $25(\text{OH})_2\text{D}_3$ and prostaglandin E_2 (PGE_2). Osteoclast precursors possess receptor activator of NF- κ B (RANK), a receptor for RANKL, and RANK-RANKL recognition induces the differentiation into mature osteoclasts.^{8,9)} Therefore, the regulation of RANKL expression in osteoblasts is thought to be a key to the treatment of various metabolic bone diseases including osteoporosis. Since the increased

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expression of RANKL has been shown in OVX rats using *in situ* hybridization,¹⁰ it is suggested that estrogen deficiency stimulates the RANK-RANKL system in bone tissues. However, it is not known how estrogen regulates the RANK-RANKL system.

In order to examine the possible relationship between the increased B-lymphopoiesis and RANKL-dependent bone resorption in estrogen deficiency, we focused on cell-to-cell interaction between bone marrow B cells and osteoblasts. In this study, we show that adhesion of B-lymphocytes to osteoblasts could induce RANKL expression in osteoblasts, suggesting a possible role of bone marrow B-lymphocytes in increased bone resorption 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). 8-week-old mice were either sham-operated or OVX. All procedures were performed in accordance with institutional guideline for animal research at the Tokyo University of Pharmacy and Life Science. PD98059 (an inhibitor of ERK1/2) and SB203580 (an inhibitor of p38) were purchased from CN Biosciences Inc (San Diego, CA, U.S.A.).

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), as reported previously.² The bone mineral content (BMC) of the mouse femur was closely correlated with the ash weight.² The BMD was calculated by dividing the BMC of the measured area by the area.

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), as described previously.¹¹ These cells were washed with PBS (–) containing 1% bovine serum albumin (BSA) and analyzed on a flow cytometer (FACS calibur, 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 with PBS (–) and resuspended in EDTA/PBS containing 0.5% BSA. B-lymphocytes were isolated from bone marrow with a magnetic cell-sorting (MACS) system using magnetic micro-beads coated with anti-mouse CD45R/B220 (Pharmingen). 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, Tokyo, Japan) and 0.2% dispase (Godo Shusei, Tokyo, Japan), as described previously.¹¹ Osteoblasts were cultured in α MEM supplemented with 10% FCS at 37°C under 5% CO₂ 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 co-cultured cells, osteoblasts and fixed-B cells, using ISOGEN (Wako). cDNA was synthesized from 5 μ g of the total RNA by reverse transcriptase (Superscript II Preamplification System, Life Technologies, Grand Island, NY, U.S.A.) and amplified using PCR. The primers used in PCR for the mouse RANKL gene were 5'-GAC TCG ACT CTG GAG AGT-3' (sense primer) and 5'-GAG AAC TTG GGA TTT TGA TGC-3' (anti-sense primer). The reaction conditions for PCR were 32 cycles, denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec, and extension at 72°C for 2 min. The primers used in PCR for the mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene were 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3' (sense primer) and 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3' (anti-sense primer). The reaction conditions for PCR were 30 cycles, denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 2 min. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. The signals were densitometrically quantified using an NIH-image analyzer.

Statistical Analysis — The data are expressed as means \pm SEM. The significance of differences was analyzed using Student's *t*-test.

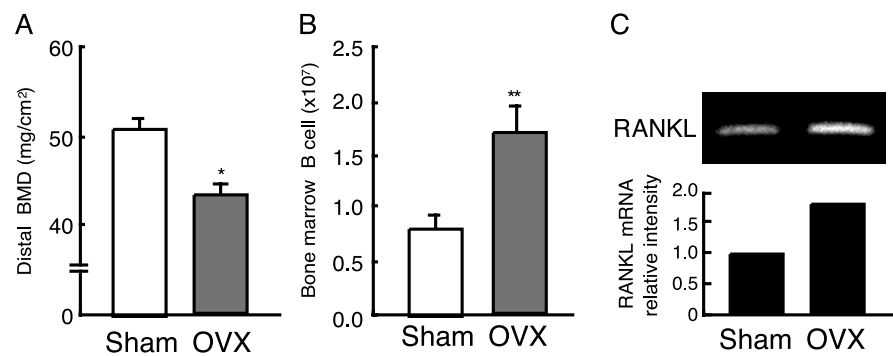


Fig. 1. Femoral BMD, the Number of Bone Marrow B Cells, and the Expression of RANKL mRNA in Trabecular Bone and Bone Marrow in Sham-Operated and OVX Mice

Eight-week-old mice were sham-operated or OVX. (A) Femur was collected 4 weeks after operation, and BMD at distal femur was measured. (B) The number of B cells in bone marrow was calculated by flow cytometric analysis using FITC-B220, 2 weeks after operation. (C) The expression of RANKL mRNA was analyzed by RT-PCR using total RNA extracted from trabecular bone and bone marrow 2 week after operation. Note that expression level of RANKL mRNA was elevated in OVX mouse compared with sham mouse. Significantly different from sham, * $p < 0.05$, ** $p < 0.01$. Data are expressed as means \pm SEM of 5–7 mice.

RESULTS AND DISCUSSION

Bone Loss, Increased B-Lymphopoiesis and Expression of RANKL in OVX Mice

Figure 1 shows the influence of OVX on bone mass, the number of B cells in bone marrow and the expression of RANKL in bone and bone marrow. As reported previously,^{1,12} OVX mice exhibited a significant decrease in femoral BMD, and an increased number of bone marrow B cells 2–4 weeks after surgery (Figs. 1A and 1B). Two weeks after surgery, total RNA was extracted from trabecular bone and bone marrow, and RT-PCR was performed to examine the expression of RANKL. Expression of RANKL mRNA could be detected in trabecular bone and bone marrow in sham-operated mice, and the level was elevated in OVX mice 2 weeks after surgery (Fig. 1C). Ikeda *et al.*¹⁰ have reported that the mRNA expression of RANKL was detected in femoral primary spongiosa in OVX rats but not in sham-operated rats suggesting that the elevated expression of RANKL in trabecular bone may contribute to the bone loss due to estrogen deficiency. However, the mechanism of elevation of RANKL expression in OVX animals is not known.

Expression of RANKL in Osteoblasts by the Contact with Fixed B-Lymphocytes

To examine the possible relationship between B cells and osteoclastic bone resorption, osteoblasts were co-cultured with B cells purified from mouse bone marrow using B220-conjugated magnetic microbeads. Since B cell weakly express RANKL

on their surface,¹³ the purified B cells were fixed with paraformaldehyde and added into the cultures of osteoblasts to examine the expression of RANKL in osteoblasts after the cell contact. As shown in Fig. 2, fixed-B cells adhered to the surface of osteoblasts time-dependent manner, and most fixed-B cells attached to osteoblast surfaces 24 hr after the addition. Various number of fixed-B cells were added to the cultures of osteoblasts, and total RNA was extracted after 24 hr. Adding fixed-B cells markedly induced the mRNA expression of RANKL in osteoblasts, and the expression levels were correlated with the number of B cells added. Since expression of RANKL mRNA was hard to detect in fixed B cells, the RANKL mRNA detected in the co-culture was resulted from osteoblasts. To examine the influence of cell-to-cell contact between B cells and osteoblasts in RANKL expression, fixed-B cells were separated from osteoblasts using a cell culture insert. The expression of RANKL mRNA 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 RANKL mRNA in osteoblasts.

To examine the mechanism of RANKL expression in osteoblasts induced by cell-to-cell contact with fixed-B cells, PD98059 (an inhibitor of ERK 1/2) and SB203580 (an inhibitor of p38 MAP kinase) were added to the co-culture of osteoblasts with fixed-B cells. Both PD98059 and SB203580 partially suppressed the expression of RANKL mRNA in osteoblasts 24 hr after the contact with fixed-B cells (Fig. 3). These results suggest the involvement of

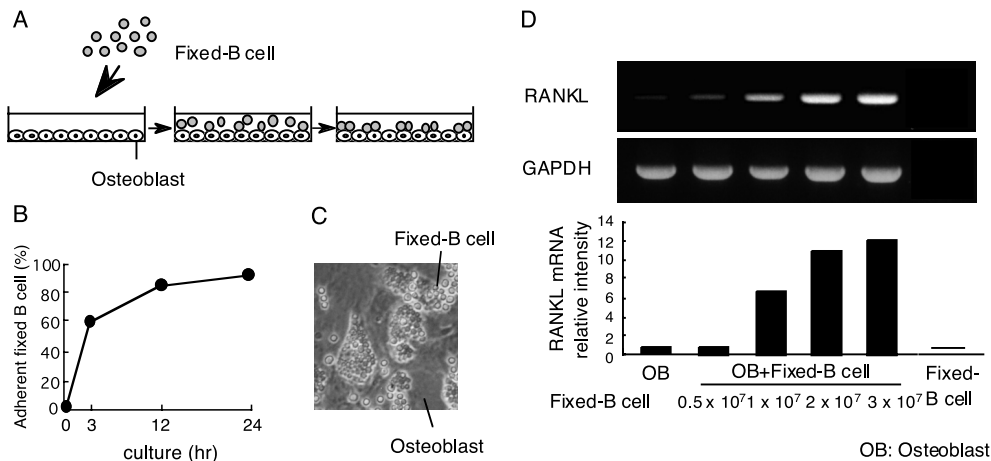


Fig. 2. The Expression of RANKL mRNA in Osteoblasts by Cell-to-Cell Contact with B-Lymphocytes

(A) 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 (2×10^7) were added to the layer of mouse osteoblasts (1×10^6) and co-cultured for 24 hr. (B) Adherent fixed-B cells was calculated from the number of fixed-B cells present in medium. Data are expressed as means \pm SEM of 3 wells. (C) Representative phase contrast image of osteoblasts and fixed-B cells co-cultured for 24 hr. (D) Various number of fixed-B cells ($0.5, 1, 2,$ and 3×10^7) were added to the layer of osteoblasts (1×10^6) in 60 mm dish and co-cultured for 24 hr. The expression of RANKL mRNA in osteoblasts, co-cultures of osteoblasts and fixed-B cells, and fixed-B cells was analyzed by RT-PCR, and relative intensity was calculated.

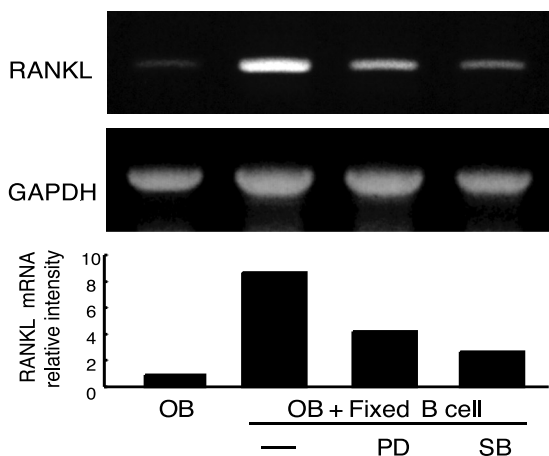


Fig. 3. Effects of PD98059 and SB203580 on the Expression of RANKL mRNA in Osteoblasts after the Contact with Fixed-B Cells

Osteoblasts were pretreated with $10 \mu\text{M}$ PD98059 and $10 \mu\text{M}$ SB203580, and then 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 to extract total RNA. The expression of RANKL mRNA was analyzed by RT-PCR, and relative intensity was calculated.

ERK and p38 MAP kinases in the signals via cell-to-cell contact between B cells and osteoblasts.

Possible Roles of B-Lymphocytes in RANKL-Dependent Bone Resorption

In this study, cell-to-cell interaction between B-lymphocytes and osteoblasts induced the expression of RANKL in osteoblasts by mechanisms involving

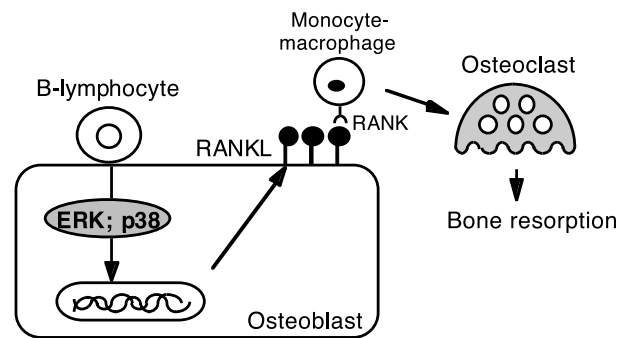


Fig. 4. Model of the Mechanism of RANKL Expression in Osteoblasts by Cell-to-Cell Contact with B-Lymphocytes
Contact of B cells with osteoblasts induces the expression of RANKL mRNA in osteoblasts by the activation of ERK1/2 and p38 MAP kinases.

MAP kinases (Fig. 4). Osteoblasts express integrins such as $\alpha 1, \alpha 2, \alpha 3, \alpha 5, \alpha v, \beta 1, \beta 3$ and $\beta 5$, and $\beta 1$ -integrin signal is involved in the activation of MAP kinase in osteoblasts.¹⁴⁾ Further studies are needed to define a possible adhesion molecule(s) involved in the cell-to-cell contact between B cells and osteoblasts.

Recently, several studies have demonstrated that myeloma cells, bone marrow plasma cells derived from patients with multiple myeloma, enhance RANKL expression by bone marrow stromal cells through direct cell-to-cell contact.¹⁵⁻¹⁷⁾ Increased expression of RANKL by bone marrow stromal cells was associated with enhanced osteoclastogenesis.¹⁷⁾ When B cells were removed from bone marrow cells,

osteoclast formation was suppressed in co-cultures of the bone marrow cells and osteoblasts (Matsumoto C. *et al.* unpublished data). Since B cells are elevated in bone marrow not only in OVX mice, but also in patients of postmenopausal osteoporosis,¹⁸⁾ the mechanism of increased osteoclastogenesis due to estrogen deficiency may be similar to that in multiple myeloma.

In conclusion, the data obtained through our present study indicate that cell-to-cell interaction between B-lymphocytes and osteoblasts could induce RANKL expression in osteoblasts, suggesting that increased B-lymphopoiesis is closely associated with bone resorption due to estrogen deficiency.

REFERENCES

- 1) Masuzawa, T., Miyaura, C., Onoe, Y., Kusano, K., Ohta, H., Nozawa, S. and Suda, T. (1994) Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. *J. Clin. Invest.*, **94**, 1090–1097.
- 2) Miyaura, C., Onoe, Y., Inada, M., Maki, K., Ikuta, K., Ito, M. and Suda, T. (1997) Increased B-lymphopoiesis by interleukin-7 induces bone loss in mice with intact ovarian function: Similarity to estrogen deficiency. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 9360–9365.
- 3) Kimble, R. B., Vannice, J. L., Bloedow, D. C., Thompson, R. C., Hopfer, W., Kung, V. T., Brownfield, C. and Pacifici, R. (1994) Interleukin-1 receptor antagonist decreases bone loss and bone resorption in ovariectomized rats. *J. Clin. Invest.*, **93**, 1959–1967.
- 4) Cenci, S., Weitzmann, M. N., Roggia, C., Namba, N., Novack, D., Woodring, J. and Pacifici, R. (2000) Estrogen deficiency induces bone loss by enhancing T-cell production of TNF- α . *J. Clin. Invest.*, **106**, 1229–1237.
- 5) Manolagas, S. C. and Jilka, R. L. (1995) Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N. Engl. J. Med.*, **332**, 305–311.
- 6) Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Poux, E. R., Teepe, M. C., DuBose, R. F., Cosman, D. and Galibert, L. (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature (London)*, **390**, 175–179.
- 7) Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F. S., Frankel, W. N., III., Lee, S. Y. and Choi, Y. (1997) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem.*, **272**, 25190–25194.
- 8) Lacey, D. L., Timms, E., Tan, H. L., Kelly, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J. and Boyle, W. J. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, **93**, 165–176.
- 9) Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinoshita, M., Mochizuki, S., Tomoyasu, A., Yan, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, N., Udagawa, N., Takahashi, N. and Suda, T. (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 3597–3602.
- 10) Ikeda, T., Utsuyama, M. and Hirokawa, K. (2001) Expression profiles of receptor activator of nuclear factor kappaB ligand, receptor activator of nuclear factor kappaB, and osteoprotegerin messenger RNA in aged and ovariectomized rat bones. *J. Bone Miner Res.*, **16**, 1416–1425.
- 11) Onoe, Y., Miyaura, C., Kaminakayashiki, T., Nagai, Y., Noguchi, K., Chen, Q.-R., Seo, H., Ohta, H., Nozawa, S., Kudo, I. and Suda, T. (1996) IL-13 and IL-14 inhibit bone resorption by suppressing cyclooxygenase-2-dependent prostaglandin synthesis in osteoblasts. *J. Immunol.*, **156**, 758–764.
- 12) Miyaura, C., Kusano, K., Masuzawa, T., Chaki, O., Onoe, Y., Aoyagi, M., Sasaki, T., Tamura, T., Koishihara, Y., Ohsugi, Y. and Suda, T. (1995) Endogenous bone-resorbing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. *J. Bone Miner Res.*, **10**, 1365–1373.
- 13) Manabe, N., Kawaguchi, H., Chikuda, H., Miyaura, C., Inada, M., Nagai, R., Nabeshima, Y., Nakamura, K., Sinclair, A. M., Scheuermann, R. H. and Kuro-o, M. (2001) Connection between B-lymphocyte and osteoclast differentiation pathways. *J. Immunol.*, **167**, 2625–2631.
- 14) Pommerenke, H., Schmidt, C., Durr, F., Nebe, B., Luthen, F., Muller, P. and Rychly, J. (2002) The mode of mechanical integrin stressing controls intracellular signaling in osteoblasts. *J. Bone Miner Res.*, **17**, 603–611.
- 15) Pearse, R. N., Sordillo, E. M., Yaccoby, S., Wong, B. R., Liao, D. F., Colman, N., Michaeli, J., Epstein, J. and Choi, Y. (2001) Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to

- trigger bone destruction and promote tumor progression. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 11581–11586.
- 16) Giuliani, N., Bataille, R., Mancini, C., Lazzaretti, M. and Barille, S. (2001) Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment. *Blood*, **98**, 3527–3533.
- 17) Roux, S., Meignin, V., Quillard, J., Meduri, G., Guiochon-Mantel, A., Ferman, J. P., Milgrom, E. and Mariette, X. (2002) RANK (receptor activator of nuclear factor-kappaB) and RANKL expression in multiple myeloma. *Br. J. Haematol.*, **117**, 86–92.
- 18) Eghbali-Fatourehchi, G., Khosla, S., Sanyal, A., Boyle, W. J., Lacey, D. L. and Riggs, B. L. (2003) Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *J. Clin. Invest.*, **111**, 1221–1230.