Journal of Cellular Biochemistry 93:503–512 (2004)

Changes in Receptor Activator of Nuclear Factor-kappaB, and Its Ligand, Osteoprotegerin, Bone-type Alkaline Phosphatase, and Tartrate-Resistant Acid Phosphatase in Ovariectomized Rats

Takashi Miyazaki,[1]* Toshiyuki Matsunaga,[1] Shuichi Miyazaki,[2] Shigeru Hokari,[1] and Tsugikazu Komoda[1]

[1]Department of Biochemistry, Saitama Medical School, 38 Morohongo Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[2]Immunology Laboratory, Yamasa Corporation, 2-10-1 Araoi-cho, Choshi, Chiba 288-0056, Japan

Abstract We investigated time-course changes in the expression of receptor activator of nuclear factor-kappaB (RANK), its ligand (RANKL), osteoprotegerin (OPG), bone-type alkaline phosphatase (BAP), and tartrate-resistant acid phosphatase (TRAP) in ovariectomized (OVX) rats. Samples of sera and coccyges were used for analysis of the enzyme activities and expression levels of proteins and mRNAs, and an immunohistochemical analysis was also performed. Serum BAP activity increased to 158.6% of the pre-operation value at 1 week after OVX, and then decreased to 38.7% at 8 weeks after OVX. On the other hand, the serum TRAP activity increased to 130.9% of the pre-operation level at 1 week after OVX, and was maintained at a high level, compared with the pre-operation level. The patterns of BAP and TRAP activity in the coccyges specimens were similar to those seen in the sera. The expression profiles of TRAP, RANK, and RANKL proteins in the coccyx specimens were similar to the pattern of serum TRAP activity, while the profiles of the BAP and OPG proteins were similar to the pattern of serum BAP activity in OVX rats. The changes in the mRNA expression levels of the osteogenic proteins were similar to those for protein expression. These biochemical changes in OVX rats were confirmed by immunohistochemical studies. Our results suggest that not only osteoclastogenesis accelerated but also osteoblastogenesis transiently increased during the early phase of osteoporosis. J. Cell. Biochem. 93: 503–512, 2004.

Key words: osteogenic proteins; bone metabolic markers; ovariectomy; rats

The development and maintenance of bone tissues is controlled by the dynamic balance between bone formation by osteoblasts and bone resorption by osteoclasts [Eriksen et al., 1985; Oursler et al., 1993]. Imbalances in these cell activities are responsible for various bone metabolic diseases. Osteoclast activation and maturation are regulated by the following three recently discovered proteins: (i) the receptor activator of nuclear factor-kappaB (RANK), expressed on the surfaces of pre-osteoclasts [Cappellen et al., 2002]; (ii) RANK ligand (RANKL), expressed on the surfaces of osteoblastic cells [Hofbauer et al., 2001]; and (iii) osteoprotegerin (OPG), a soluble decoy receptor for RANKL that is expressed in osteoblasts [Bu et al., 2003]. The binding of RANKL to RANK on pre-osteoclasts, initiates the differentiation and proliferation of these cells, and promotes osteoclast fusion and activation. Moreover, the activation of the RANKL-RANK pathway suppresses osteoclast apoptosis, resulting in an increase the number of activated osteoclasts [Hofbauer et al., 2001; Cappellen et al., 2002]. On the other hand, OPG inhibits this pathway by binding to RANKL [Bu et al., 2003]. Thus, the above-mentioned proteins play essential roles in the development and maintenance of bone tissues.

Ovarian hormones are well known to influence the maintenance of bone tissues [Lund et al., 1982]. Ovarian hormone deficiency is the most important risk factor for osteoporosis

*Correspondence to: Takashi Miyazaki, Department of Biochemistry, Saitama Medical School, 38 Morohongo Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan.
E-mail: miyasan@saitama-med.ac.jp
Received 2 March 2004; Accepted 14 May 2004
DOI 10.1002/jcb.20201

[Lund et al., 1982], a disease that is characterized by increased bone loss soon after natural or surgical menopause in women [Yildiz et al., 1996]. Ovariectomized (OVX) rat is a useful animal model for osteoporosis caused by estrogen deficiency [Ammann et al., 1992]. This model exhibits a progressive loss of bone matrix through a process that is similar to what occurs during postmenopausal osteoporosis [Heaney et al., 1978; Stepan et al., 1987; Ammann et al., 1992].

Bone-type alkaline phosphatase (BAP) and tartrate-resistant acid phosphatase (TRAP) are well-established metabolic markers of osteoblastic bone formation and osteoclastic bone resorption, respectively [Garnero and Delmas, 1996; Halleen et al., 2001; Miyazaki et al., 2003]. Modrowski and Marie [1993] reported that the proliferation rates of BAP-positive osteoblasts and the serum enzyme activities of BAP and TRAP were increased by OVX [Tanizawa et al., 2000]. Moreover, Kanematsu et al. [2000] and Nielsen et al. [2003] reported that role of the RANKL/OPG was studied in pre-B cell from OVX mice and in serum of 21-week-old biglygan knockout mice, respectively. However, few, if any, studies have investigated time-course changes in the expression levels of osteogenic proteins and mRNAs and the dynamic changes in osteoblasts, pre-osteoclasts, and osteoclasts.

To clarify the involvement of RANK, RANKL, and OPG on bone metabolism, we investigated time-course changes in their protein and mRNA expression patterns using Western blotting and reverse transcription-polymerase chain reaction (RT-PCR), respectively in OVX rats. In addition, time-course changes in TRAP and BAP expression, two traditional markers of bone metabolism were examined, and dynamic changes in osteogenic cells were studied using immunohistochemical analyses.

MATERIALS AND METHODS

Antibodies and Chemicals

Antibodies against mouse RANK, mouse RANKL, and mouse OPG were kindly purchased from Sankyo Co. (Tokyo, Japan) as in previous studies [Nakagawa et al., 1998; Tsukii et al., 1998; Yano et al., 2001]. Anti-human TRAP antibody was obtained from Yamasa Corporation (Choshi, Japan) [Miyazaki et al., 2002]. The anti-rat BAP antibody was a kind gift from Prof. DH Alpers (Washington University School of Medicine, St. Louis). Other chemicals used in this study were purchased from Sigma Chemical Co., (St. Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Ovariectomy

Eight-week-old female Wistar rats (Clea Japan, Tokyo, Japan) with a mean body weight of 180 g were assigned to control and two groups: OVX ($n = 30$), sham-operation ($n = 12$), and pre-operation (control $n = 6$). Rats in the two groups were subjected to a bilateral OVX or sham operation via a dorsal approach under an intraperitoneal injection of Nembutal® (Abbot Laboratories, North Chicago, IL), according to the procedure described by Wronski et al. [1985]. The success of the OVX operation was confirmed by necropsy, i.e., the absence of ovarian tissue and the presence of marked uterine horn atrophy. In the pre-operation rats, they were sacrificed under deep ether anesthesia at the start of the experiment, and the coccyges (2nd–5th caudal vertebrae) were excised. In the OVX ($n = 5$ for each time point) and sham-operation ($n = 2$ each time point) groups, the coccyges were excised 1, 2, 3, 4, 5, or 8 weeks after the operation. All specimens were stored at $-80^{\circ}C$ until analysis. Blood samples were obtained by left ventricular puncture, and the serum was separated by centrifugation. All experimental protocols were approved by the Animal Research Committee of Saitama Medical School prior to the start of the experiments.

Assay of BAP and TRAP Activities in the Sera and Coccyges

The enzyme activities of phosphatase were assayed using a previously described method [Fujimori-Arai et al., 1991; Nosjean et al., 1997]. Serum BAP was estimated using an ALP isozymes PAG disc electrophoresis kit (AlkPhor system; Jokoh, Tokyo, Japan). Bone extracts were prepared according to Miyazaki et al. [2003]. Briefly, after removal of all connective tissue from coccyges, 50–100 mg of these were crushed by surgical pliers, homogenized with BAP buffer (50 mM Tris-Hcl buffer, pH 7.5, containing 0.3 mM phenylmethyl fluoride, 1.0 mM benzamidine, and 0.1% Triton X-100) or TRAP buffer (BAP buffer containing 200 mM KCl), and the homogenate was centrifuged at 12,000*g* for 20 min at $4^{\circ}C$. Resultant supernatant was used for enzyme assays and Western

blot analysis. Protein contents were determined using a BCA Protein assay kit (PIERCE, Rockford, IL) using bovine serum albumin as the standard. Phosphatase activity was expressed as U/ml and U/mg in the serum and coccyx extracts, respectively [Fujimori-Arai et al., 1991; Nosjean et al., 1997]. One unit of enzyme activity was defined as that required to hydrolyze 1 μmol of substrate/min at 37°C.

Western Blot Analysis

Western blotting was performed using a previously described method [Fujimori-Arai et al., 1991; Nosjean et al., 1997]. Briefly, proteins separated by electrophoresis were transferred to a polyvinylidene difluoride membrane filter (Millipore, Tokyo, Japan). After the filter was blocked by Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) for 120 min, the proteins on the membrane were incubated with anti-RANK, anti-RANKL, anti-OPG, anti-BAP, or anti-TRAP antibodies for overnight at 4°C. After washing, the filter was reacted with peroxidase-conjugated secondary antibodies for 120 min at room temperature. The reactive bands were detected with the ECL system (Amersham Biosciences, Co., Piscataway, NJ), and visualized bands were evaluated by densitometric analysis using an ATTO Densitograph (ATTO, Tokyo, Japan). The relative intensities from pre-operation level were calculated and expressed by percent change.

RNA Preparation and RT-PCR

Total RNA was prepared from the coccyges samples using the acid guanidium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Briefly, 0.5 μg of total RNA prepared was added to a Titan™ One Tube RT-PCR system (Roche Diagnostics GmbH, Mannheim, Germany). The RT reaction was carried out at 50°C for 30 min, and then the transcriptase (avian myeloblastosis virus transcriptase) was inactivated at 94°C for 5 min. PCR was performed using the GeneAmp PCR system 9700 (Perkin-Elmer Life Science, Inc., Wellesley, MA); 33 cycles of amplification were performed for RANKL, OPG, BAP, TRAP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 36 cycles were performed for RANK under the following amplification conditions; (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 1 min). The forward (F) and reverse (R) primer sequences used to detect the individual mRNA and the product sizes are shown in Table I.

TABLE I. Primer Sequences for the Detection of Osteogenic Protein mRNAs

Products	Sequences
RANK (497 bp)	F; 5′-ttaagccagtgcttcacggg-3′ R; 5′-acgtagaccacgatgatgtcgc-3′
RNKL (407 bp)	F; 5′-acaggttt gcaggactcgactg-3′ R; 5′-aggtacgctccctgaggtttca-3′
OPG (480 bp)	F; 5′-accctgtgcgaagaggcattctt-3′ R; 5′-gtgcaagaacctgatggtcttc-3′
BAP (327 bp)	F; 5′-aagtccgtgggcatcgtgac-3′ R; 5′-gtgggagtgcttgtgtctag-3′
TRAP (463 bp)	F; 5′-agatctccaagcgctggaac-3′ R; 5′-aggtagccgttggggacctt-3′
GAPDH (452 bp)	F; 5′-accacagtccatgccatcac-3′ R; 5′-tccaccaccctgttgctgta-3′

Forward (F) and reverse (R) primer used for detection of the individual mRNA. Numbers in parentheses represent the product sizes.

The PCR products were separated by electrophoresis on a 5% polyacrylamide slab gel and then visualized by staining with ethidium bromide and evaluated densitometrically [Fujimori-Arai et al., 1991; Nosjean et al., 1997]. The expression levels of RANK, RANKL, OPG, BAP, and TRAP mRNA were normalized according to the GAPDH level. The relative intensities from pre-operation level (1.0) were calculated and expressed.

Histochemistry and Immunohistochemistry Analyses for RANK, BAP, and TRAP

The histochemical and immunohistochemical procedures in the rat bone tissues were performed as described previously [Miyazaki et al., 2002]. Immunoreactivity was visualized by diaminobenzidine and counterstained with hematoxylin. Osteoclasts were identified by the presence of multiple nuclei (more than three) in cells that stained positively with anti-TRAP antibody [Miyazaki et al., 2002]. Pre-osteoclasts [Cappellen et al., 2002] were defined as anti-RANK antibody-positive cells or osteoblasts as anti-BAP antibody-positive cells [Murray et al., 1989]. Cell numbers were counted under a light microscope at a magnification of 100×, and the ratio of positive cells to the total number of cells was estimated using the average value for five visual fields.

Statistical Analysis

The statistical analysis was performed using the non-parametric Kruskal–Wallis/Mann–

Whitney *U*-test. Data are expressed as the mean ± SEM.

RESULTS

Enzyme Activities of BAP and TRAP in Serum and Coccyx

The serum BAP activity level increased significantly to $1.76 \pm 0.16 \times 10^{-3}$ U/ml at 1 week after OVX, compared with $1.11 \pm 0.18 \times 10^{-3}$ U/ml pre-operation, then decreased to $0.43 \pm 0.15 \times 10^{-3}$ U/ml at 8 weeks after OVX (Fig. 1A). In the sham group, the serum BAP activity level gradually decreased to $0.39 \pm 0.15 \times 10^{-3}$ U/ml at 8 weeks after operation, compared with $1.17 \pm 0.15 \times 10^{-3}$ U/ml pre-operation.

The serum TRAP activity level increased significantly to 0.014 ± 0.002 U/ml at 1 week after the OVX compared with 0.012 ± 0.001 U/ml pre-operation, and peaked (0.017 ± 0.002 U/ml) at 4 weeks after the OVX (Fig. 1B). In the sham group, the serum TRAP activity level slightly increased from the pre-operation level throughout the experimental period.

BAP activity level in the coccygeal extracts increased significantly to 1.52 ± 0.12 U/mg at 2 weeks after the OVX, compared with pre-operation (1.25 ± 0.10 U/mg), and then decreased to 0.87 ± 0.05 U/mg at 8 weeks after the OVX (Fig. 2A). BAP activity in the sham group decreased to 0.90 ± 0.08 U/mg at 8 weeks after the operation compared with pre-operation (1.28 ± 0.1 U/mg).

TRAP activity level in the coccygeal extracts increased significantly to 0.40 ± 0.04 U/mg at 2 weeks after the OVX, compared with pre-operation (0.32 ± 0.03 U/mg), then peaked at 4 weeks after the OVX, and was maintained until 8 weeks after the OVX. TRAP activity in the sham group slightly increased from the pre-operation level throughout the experimental period (Fig. 2B).

Changes in the Levels of BAP, TRAP, RANK, RANKL, and OPG Protein in the Coccyges of Rats

The BAP protein (72 kDa) level at 1 week after OVX was significantly higher than pre-operation level, and peaked at 3 weeks after the OVX, 227.2 ± 28.3% of the pre-operation level, then decreased to 128.2 ± 18.7% at 8 weeks after the OVX ((Fig. 3A(a) and 3C).

The TRAP protein (36 kDa) level at 1 week after the OVX was significantly higher than the pre-operation level (Fig. 3A(c)), and peaked at 3 weeks after the OVX (400.2 ± 45.6% of the pre-operation level), then decreased thereafter (Fig. 3C).

The RANK protein (62 kDa) level at 1 week after the OVX was significantly higher than the pre-operation level (Fig. 3B(a)) and peaked at

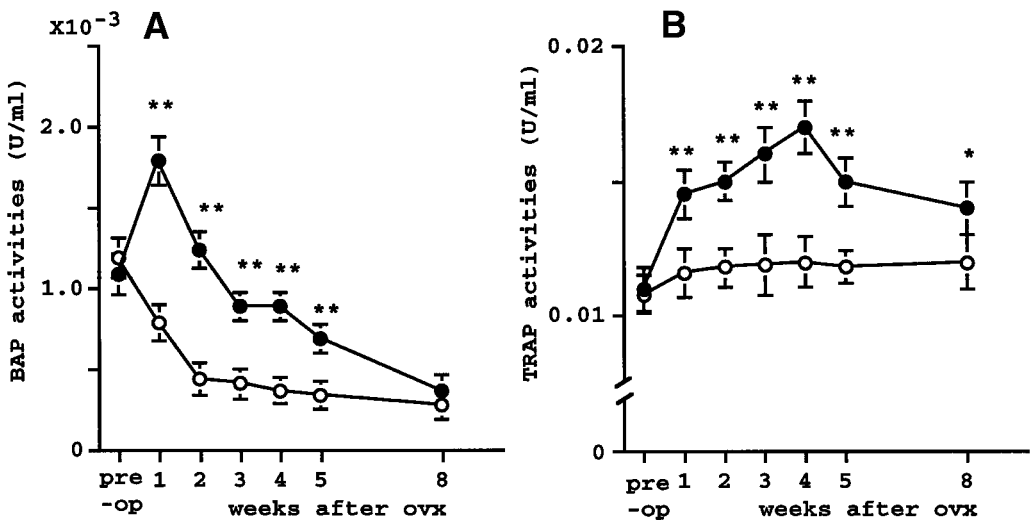

Fig. 1. Changes in phosphatase activities in sera of rats after operation. **A**: Bone-type alkaline phosphatase (BAP) activities are shown in the ovariectomized (OVX) group (closed circles) and the sham group (open circles) from pre-op (pre-operation) to 8 weeks after operation. **B**: Tartrate-resistant acid phosphatase (TRAP) activities are shown in the OVX group (closed circles) and sham group (open circles). Enzyme activities were assayed as described in the "Materials and Methods" section. Data are shown as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus corresponding time point in the sham group. Data are calculated from five experiments (OVX group) or three experiments (sham group) in triplicate measurements of each sample.

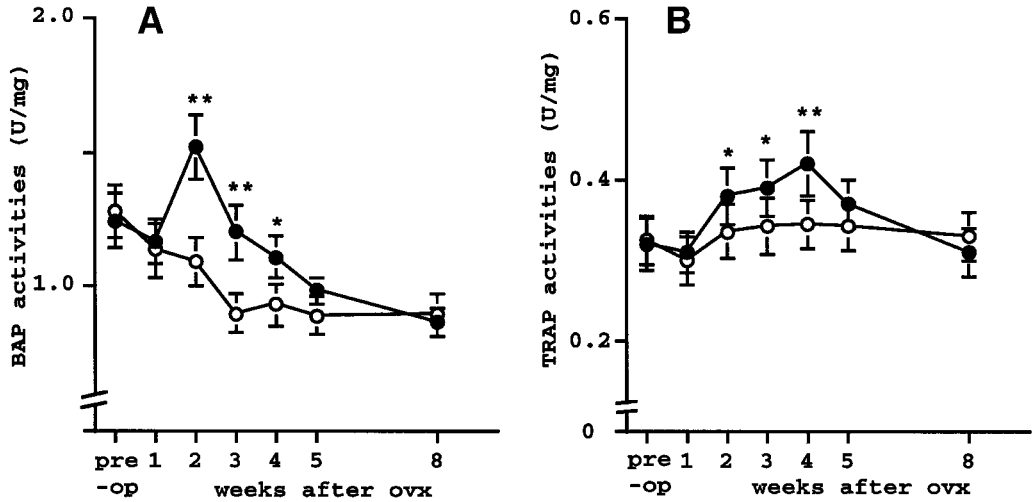

Fig. 2. Changes in phosphatase activities in the coccyges of rats after operation (**A**) BAP activities are shown in the OVX group (closed circles) and the sham group (open circles) from pre-op (pre-operation) to 8 weeks after OVX. **B**: TRAP activities are shown in the OVX group (closed circles) and the sham group (open circles). Enzyme activities were assayed as described in the "Materials and Methods" section. Data are shown as the mean ± SEM. $*P < 0.05$, $**P < 0.01$ versus corresponding time point in the sham group. Data are calculated from five experiments (OVX group) or three experiments (sham group) in triplicate measurement of each sample.

4 weeks after the OVX (230.2 ± 31.1% of the pre-operation level), this level was maintained up to 8 weeks after OVX (Fig. 3D).

RANKL protein (40 kDa) level was significantly increased at 1 week after OVX comparing to the pre-operation level (Fig. 3B(c),D), peaked at 5 weeks (294.1 ± 33.4% of pre-operation level), and this level was maintained until 8 weeks after the OVX (Fig. 3D).

The OPG protein (59 kDa) level at 1 week after the OVX was significantly higher than the pre-operation level (Fig. 3B(e)) and peaked at 2 weeks after the OVX (269.4 ± 31.5% of the pre-operation level), then gradually decreased to 142.3 ± 19.2% at 8 weeks after the OVX (Fig. 3D).

All of the protein levels tested in the sham group did not change throughout the experimental period (Fig. 3).

Changes in mRNA Levels of BAP, TRAP, RANK, RANKL, and OPG in the Coccyges of Rats

The RT-PCR products of BAP, TRAP, RANK, RANKL, and OGP were clearly detected using the PCR conditions described in the "Materials and Methods" section. The level of the target product was normalized against the level of a house keeping gene product, GAPDH (Fig. 4). The BAP mRNA expression level increased to 5-fold at 3 weeks, compared with the pre-operation level, then gradually decreased to 1.5-fold at 8 weeks after the OVX.

The TRAP mRNA expression level increased to 4-fold at 3 weeks compared with the pre-operation level, and this level was maintained until 8 weeks after the OVX.

RANK and RANKL mRNA expression levels increased to 5.6- and 4.6-fold at 3 weeks, compared with the pre-operation level, respectively. These levels were maintained until 8 weeks after the OVX.

The OPG mRNA expression level increased to 4.6-fold at 3 weeks, compared with the pre-operation level, then decreased to 1.8-fold at 8 weeks after the OVX.

The mRNA levels in the sham group did not change throughout the experimental period (data not shown).

Histochemistry and Immunohistochemistry Analyses for BAP, RANK, and TRAP Expression

Histochemical analyses of the bone tissues were performed on samples collected from the pre-operation rats and in samples collected from the OVX and sham group rats. Time-related cancellous bone loss was observed in the OVX group, but not in the sham group (data not shown).

In the immunohistochemical analysis, BAP-, RANK-, and TRAP-positive cells were observed not only in the specimens from the pre-operation rats, but also in those from the OVX group (Fig. 5A–I). However, OPG and RANKL-

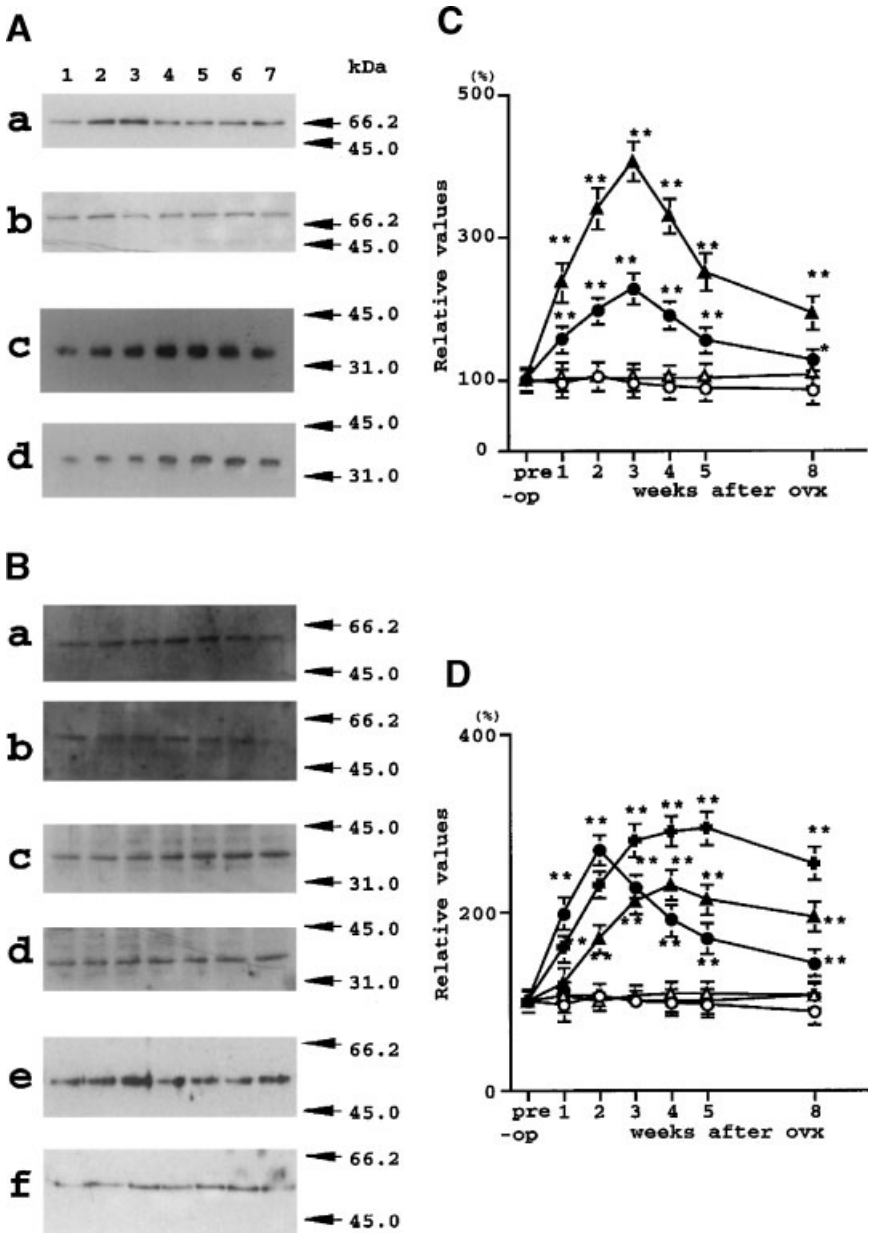

Fig. 3. Time course changes in osteogenic protein levels in the coccyges of rats. Quantitative analyses of BAP, TRAP, RANK, RANKL, and OPG proteins were performed as described in the ''Materials and Methods.'' **A**: BAP proteins in the OVX group (**a**) and the sham group (**b**), and TRAP proteins in the OVX group (**c**) and the sham group (**d**) were analyzed by Western blotting. **Lanes 1–7** corresponded to the samples obtained before pre-operation and 1, 2, 3, 4, 5, and 8 weeks after operation. **B**: RANK proteins in the OVX group (**a**) and the sham group (**b**), RANKL proteins in the OVX group (**c**) and the sham group (**d**), OPG proteins in the OVX group (**e**), and the sham group (**f**) were detected. Changes of these osteogenic proteins from pre-operation level (100%) were quantitatively evaluated by densitometric analysis. **C**: Changes in BAP protein levels in the OVX group (closed circles) and the sham group (open circles), TRAP protein levels in the OVX group (closed triangles) and the sham group (open triangles). **D**: Changes in RANK proteins in the OVX group (closed triangles) and the sham group (open triangles), RANKL protein levels in the OVX group (closed crosses) and the sham group (open crosses), and OPG protein levels in the OVX group (closed circles) and the sham group (open circles). Other experimental conditions are as described in the ''Materials and Methods'' section. Data are shown as the mean ± SEM. $*P < 0.05$, $**P < 0.01$ versus corresponding time point in the sham group. Data are calculated from five experiments (OVX group) or three experiments (sham group) in triplicate measurements of each sample.

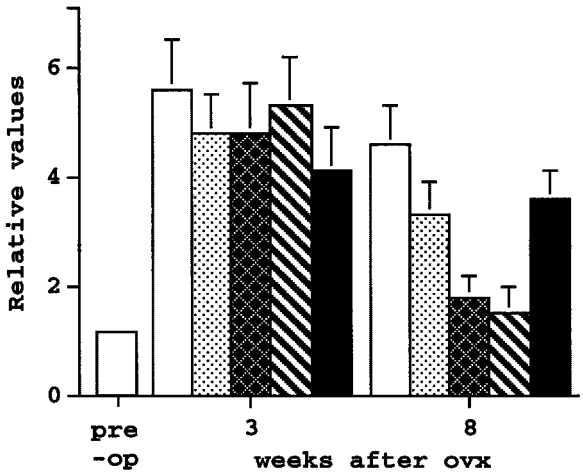

Fig. 4. Expression of mRNAs for osteogenic proteins in the coccyges of rats belonging to the OVX group The relative values of mRNA expression (RANK/GAPDH, open column; RANKL/GAPDH, hatched column; OPG/GAPDH, meshed column; BAP/GAPDH, slashed column; and TRAP/GAPDH, closed column) from pre-operation level (1.0) were expressed. Other experimental conditions are as described in the "Materials and Methods" section. Data are shown as the mean ± SEM versus pre-operation level, and are calculated from five experiments (OVX group) or three experiments (sham group) in triplicate measurement of each sample.

positive cells were not detected in the present study. The number of BAP-positive cells (osteoblasts; Fig. 5A–C), RANK-positive cells (pre-osteoclasts; Fig. 5D–F), and TRAP-positive cells (osteoclasts; Fig. 5G–I) had increased at 3 weeks after the OVX, compared with the pre-operation level. However, the number of osteoblasts was much lower than pre-osteoclasts number at 8 weeks after the OVX.

The ratio of osteoblasts to the total cell number increased significantly to $23.5 \pm 2.5\%$ at 3 weeks, compared with $8.5 \pm 0.8\%$ of pre-operation, and then decreased to $14.8 \pm 2.6\%$ at 8 weeks after the OVX (Fig. 6).

The ratio of pre-osteoclasts also increased significantly to $22.1 \pm 2.5\%$ at 3 weeks, compared with $10.8 \pm 1.2\%$ of pre-operation, and then decreased to $15.6 \pm 1.5\%$ at 8 weeks after the OVX.

The ratio of the osteoclasts increased significantly to $4.8 \pm 0.3\%$ at 3 weeks, compared with $3.3 \pm 0.3\%$ of pre-operation, and was maintained until 8 weeks after OVX.

The ratio of above cells in the sham group did not change from the pre-operation levels throughout the experimental period (data not shown).

DISCUSSION

In the present study, we investigated the time-course changes in potential bone metabolic markers, RANKL, RANK, and OPG, in addition to traditional markers, BAP and TRAP, using biochemical and histochemical methods in OVX rat. Our results revealed that all of the examined biochemical markers were activated by OVX. However, the time-course changes in their mRNA and protein expression levels differed somewhat. Changes in bone formation markers, BAP and OPG, were transient, while changes in bone resorption markers, TRAP, RANK, and RANKL, were persistent. These results were confirmed by an immunohistochemical study, in which an increase in osteoblastic cells was only transiently observed during the early phase after OVX, while the increase in activated osteoclasts was persistent.

The profile of BAP activity in sham group was different from result of Sims et al. [1996]. Since, they used 6-month-old (25–26 weeks) rats and we used 8 week-old rats, young rat's serum BAP activity may have a higher than old rat's as seen in a healthy children [Tobiume et al., 1997].

The formation and activation of osteoclast depend on the expression of RANKL on the osteoblast's surface [Nakagawa et al., 1998; Tsukii et al., 1998]. Therefore, one of the mechanisms responsible for the increase in osteoclast cells may be an increase in the osteoblast number caused by the activation of the RANKL-RANK pathway in the early phase after OVX. Furthermore, the turnover of osteoclastogenesis and osteoblastogenesis is reportedly disturbed by osteoporosis [Heaney et al., 1978; Lund et al., 1982; Stepan et al., 1987; Ammann et al., 1992; Yildiz et al., 1996]. Since this imbalance may progress slowly after natural menopause, the initiator of this process might be associated with a transient increase in the osteoblastogenesis seen in the present rat model for osteoporosis. Another mechanism of the imbalance between osteoclastogenesis and osteoblastogenesis might involve an inhibition of osteoclast apoptosis or an acceleration of osteoblast apoptosis. Using cell survival and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay, Duque et al. [2002] reported that the supplementation of 17β-estradiol to human osteoblast reduced apoptosis. Osteoclast apoptosis is stimulated by 17β-estradiol through the production of TGF-β-1 on osteoblasts [Hughes

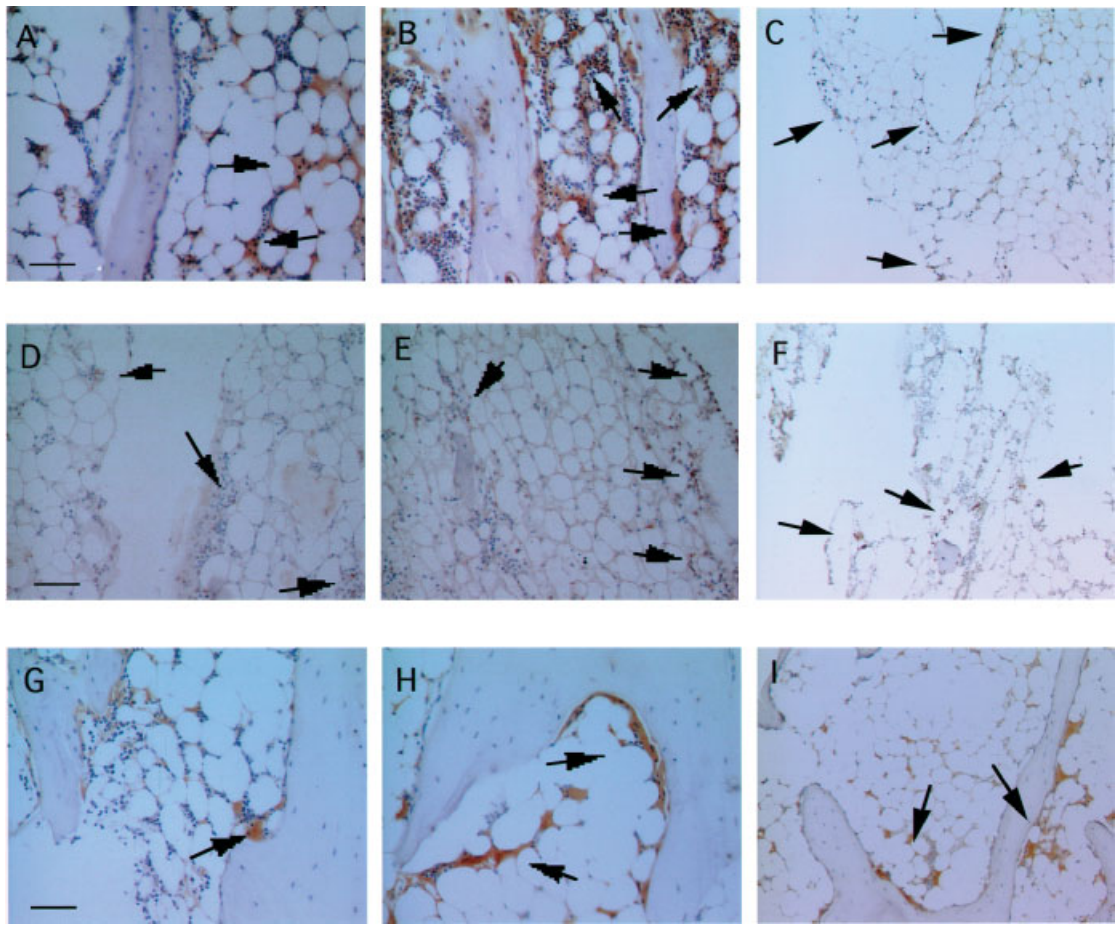

Fig. 5. Histochemistry and immunohistochemistry analyses in the coccyges of rats Time-course changes in coccyx sections of specimens are shown. BAP-positive (**A**, **B**, and **C**), with RANK-positive (**D**, **E**, and **F**), and with TRAP-positive (**G**, **H**, and **I**) sections are shown. Pre-operation group (A, D and G), OVX group after 3 weeks (B, E and H), and 8 weeks (C, F and I). The arrows indicate the positively stained cells. The length of the bar in the photographs is 100 μm. Other experimental conditions are as described in the ''Materials and Methods'' section.

et al., 1996]. In fact, we observed that serum 17β-estradiol was quickly depleted after OVX (unpublished observations). Therefore, the estrogen depletion caused by the OVX might directly affect the acceleration of osteoblast apoptosis or the inhibition of osteoclast apoptosis.

In histochemical analyses, the rat coccyx is superior to other spinal bones for the monitoring of bone mineral content in vivo because the coccyx is connected to the vertebrae but the mechanical features of the coccyx (1st–5th on proximal side of lumbar vertebra) are similar to lumbar vertebrae. Li et al. [1996] also reported that the bone turnover rate of the 5th coccyx was increased, relative to that in control rats at 30 days after OVX. However, Miyakoshi et al. [1999] performed bone histomorphometric evaluations of the coccyges (1st, 3rd, and 5th) and lumbar vertebrae (L4) in OVX rats (7-month-old Wistar rats) and reported that the yellow marrow-enriched 3rd and 5th coccyges did not exhibited osteopenia, but red marrow enriched L4 vertebrae and 1st coccyges did exhibit osteopenia after OVX. We used the coccyges (2nd–5th) of 8-week-old rats, while they used 7-month-old rats. The bone of immature rats may be more susceptible to estrogen depletion than that of mature rats.

The mean life of Japanese women is 84-years-old (data for 2001), while that of the rat is approximately 18–20 months. Using a mathematical correction, 1 week in the life of a rat corresponds to about 1 year in the life of a woman. Since transient increases in serum BAP activity were observed during 1 week after OVX in rats, this phenomenon might be also observed during the first year after menopause in women. Furthermore, since the expression profile of

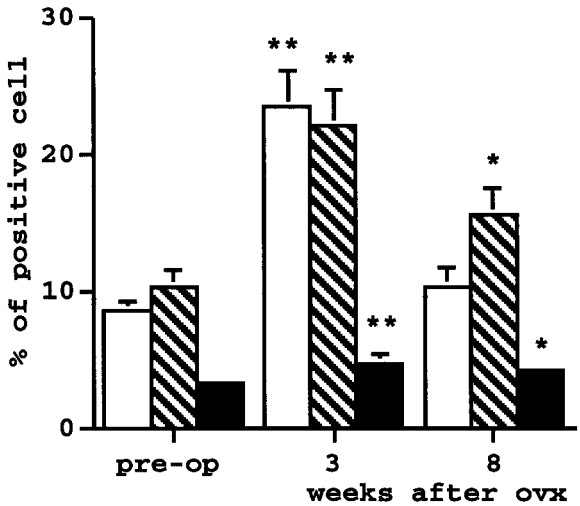

Fig. 6. Ratios of osteoblastic cells, pre-osteoclastic cells, and osteoclastic cells in the coccyges of OVX rats The ratios of cells positively stained with anti-BAP (open column; osteoblasts), anti-RANK (striped column; pre-osteoclasts) and anti-TRAP (closed column; osteoclasts) antibodies were evaluated in the pre-operation (pre-op) and after 3 and 8 weeks in the OVX group. Data are shown as the percentages of positive cells to the total cell number. Other experimental conditions are described in the "Materials and Methods." Values are shown as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus pre-operation values.

OPG protein was similar to that of BAP, measurement of serum OPG might be useful for the early detection of osteoporosis. Although, several bone metabolic markers are known until now [Morris et al., 1992; Modrowski and Marie, 1993; Garnero and Delmas, 1996; Halleen et al., 2001; Miyazaki et al., 2003], OPG may also be early bone metabolic marker.

Finally, the present studies suggest that a transient increase in osteoblastogenesis occurs during the early phase of osteoporosis. Medications for osteoporosis might be more effective if they are initiated during this transient increase in osteoblastogenesis.

REFERENCES

Ammann P, Rizzoli R, Slosman D, Bonjour JP. 1992. Sequential and precise in vivo measurement of bone mineral density in rats using dual-energy X-ray absorptiometry. J Bone Miner Res 7:311–316.

Bu R, Borysenko CW, Li Y, Cao L, Sabokbar A, Blair HC. 2003. Expression and function of TNF-family proteins and receptors in hsuman osteoblasts. Bone 33:760–770.

Cappellen D, Luong-Nguyen NH, Bongiovanni S, Grenet O, Wanke C, Susa M. 2002. Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NF kappa B. J Biol Chem 277: 21971–21982.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Duque G, Abdaimi KE, Macoritto M, Miller MM, Kremer R. 2002. Estrogens (E2) regulate expression and response of 1,25-dihydroxyvitamin D3 receptors in bone cells: Changes with aging and hormone deprivation. Biochem Biophys Res Commun 299:446–454.

Eriksen EF, Mosekilde L, Melsen F. 1985. Trabecular bone remodeling and bone balance in hyperthyroidism. Bone 6:421–428.

Fujimori-Arai Y, Koyama I, Hirano K, Sakagishi Y, Komoda T. 1991. Purification and partial characterization of intestinal-like alkaline phosphatase in rabbit kidney. Arch Biochem Biophys 284:320–325.

Garnero P, Delmas PD. 1996. New developments in biochemical markers for osteoporosis. Calcif Tissue Int 59:2–9.

Halleen JM, Alatalo SL, Janckila AJ, Woitge HW, Seibel MJ, Vaananen HK. 2001. Serum tartrate-resistant acid phosphatase 5b is a specific and sensitive marker of bone resorption. Clin Chem 47:597–600.

Heaney RP, Recker RR, Saville PD. 1978. Menopausal changes in bone remodeling. J Lab Clin Med 92:964–970.

Hofbauer LC, Shui C, Riggs BL, Dunstan CR, Spelsberg TC, O'Brien T, Khosla S. 2001. Effects of immunosuppressants on receptor activator of NF-kappaB ligand and osteoprotegerin production by human osteoblastic and coronary artery smooth muscle cells. Biochem Biophys Res Commun 280:334–339.

Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. 1996. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. Nat Med 2:1132–1136.

Kanematsu M, Sato T, Takai H, Watanabe K, Ikeda K, Yamada Y. 2000. Prostaglandin E2 induces expression of receptor activator of nuclear factor-kB ligand/osteoprotegerin ligand on pre-B cells: Implications for accelerated osteoclastogenesis in estrogen deficiency. J Bone Miner Res 15:1321–1329.

Li M, Shen Y, Qi H, Wronski TJ. 1996. Comparative study of skeletal response to estrogen depletion at red and yellow marrow sites in rats. Anatomical Record 245:472–480.

Lund B, Sorensen OH, Lund B, Agner E. 1982. Serum 1,25-dihydroxyvitamin D in normal subjects and in patients with postmenopausal osteopenia. Influence of age, renal function and oestrogen therapy. Horm Metab Res 14: 271–274.

Miyakoshi N, Sato K, Abe T, Tsuchida T, Tamura Y, Kudo T. 1999. Histomorphometric evaluation of the effects of ovariectomy on bone turnover in rat caudal vertebrae. Calcif Tissue Int 64:318–324.

Miyazaki T, Matsunaga T, Miyazaki S, Hokari S, Komoda T. 2002. Characterization of four monoclonal antibodies to recombinant human tartrate-resistant acid phosphatase. Hybrid Hybridomics 21:191–195.

Miyazaki S, Igarashi M, Nagata A, Tominaga Y, Onodera K, Komoda T. 2003. Development of immunoassays for type-5 tartrate-resistant acid phosphatase in human serum. Clin Chim Acta 329:109–115.

Modrowski D, Marie PJ. 1993. Cells isolated from the endosteal bone surface of adult rats express differentiated osteoblastic characteristics in vitro. Cell Tissue Res 271:499–505.

Morris HA, Porter SJ, Durbridge TC, Moore RJ, Need AG, Nordin BE. 1992. Effects of oophorectomy on biochemical

and bone variables in the rat. Bone Miner 18:133–134.

Murray E, Martin E, Burton D, Deftos LJ. 1989. Rapid, simple identification of individual osteoblastic cells and their specific products by cell blotting assay. J Bone Miner Res 4:831–838.

Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T, Higashio K. 1998. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophy Res Commun 253:395–400.

Nielsen KL, Allen MA, Bloomfield SA, Andersen TL, Chen XD, Poulsen HS, Young MF, Heegaard AM. 2003. Biglycan deficiency interferes with ovariectomy-induced bone loss. J Bone Miner Res 18:2152–2158.

Nosjean O, Koyama I, Goseki M, Roux B, Komoda T. 1997. Human tissue non-specific alkaline phosphatases: Sugar-moiety-induced enzymic and antigenic modulations and genetic aspects. Biochem J 321:297–303.

Oursler MJ, Landers JP, Riggs BL, Spelsberg TC. 1993. Oestrogen effects on osteoblasts and osteoclasts. J Ann Med 25:361–371.

Sims NA, Morris HA, Moore RJ, Durbridge TC. 1996. Increased bone resorption precedes increased bone formation in the ovariectomized rat. Calcif Tissue Int 59:121–127.

Stepan JJ, Pospichal J, Presl J, Pacovsky V. 1987. Bone loss and biochemical indices of bone remodeling in surgically induced postmenopausal women. Bone 8:279–284.

Tanizawa T, Yamaguchi A, Uchiyama Y, Miyaura C, Ikeda T, Ejiri S, Nagal Y, Yamato H, Murayama H, Sato M, Nakamura T. 2000. Reduction in bone formation and elevated bone resorption in ovariectomized rats with special reference to acute inflammation. Bone 26:43–53.

Tobiume H, Kanzaki S, Hida S, Ono T, Moriwaki T, Yamauchi S, Tanaka H. 1997. Serum bone alkaline phosphatase isozymes levels in normal children and children with growth hormone (GH) deficiency: A potential marker for bone formation and response to GH therapy. J Clin Endocrinol Metab 82:2056–2061.

Tsukii K, Shima N, Mochizuki S, Yamaguchi K, Kinosaki M, Yano K, Shibata O, Udagawa N, Yasuda H, Suda T, Higashio K. 1998. Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha 25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenvironment of bone. Biochem Biophy Res Commun 246:337–341.

Wronski TJ, Lowry PL, Walsh CC, Ignaszewski LA. 1985. Skeletal alterations in ovariectomized rats. Calcif Tissue Int 37:324–328.

Yano K, Shibata O, Mizuno A, Kobayashi F, Higashio K, Morinaga T, Tsuda E. 2001. Immunological study on circulating murine osteoprotegerin/osteoclastogenesis inhibitory factor (OPG/OCIF): Possible role of OPG/OCIF in the prevention of osteoporosis in pregnancy. Biochem Biophy Res Commun 288:217–224.

Yildiz A, Sahin I, Gol K, Taner Z, Uluturk A, Biberoglu K. 1996. Bone loss rate in the lumbar spine: A comparison between natural and surgically induced menopause. Int J Gynaecol Obstet 55:153–159.