Jpn. J. Pharmacol. 80, 67-74 (1999)

Inhibitory Effect of Vitamin K_2 (Menatetrenone) on Bone Resorption in Ovariectomized Rats: A Histomorphometric and Dual Energy X-Ray Absorptiometric Study

Yasuhiro Akiyama[1], Kuniko Hara[1], Masatoshi Kobayashi[1], Takashi Tomiuga[1] and Tetsuya Nakamura[2]

[1]Department of Applied Drug Research, [2]Vitamin E Information and Technology Section, Eisai Co., Ltd., Koishikawa 4-6-10, Bunkyo-ku, Tokyo 112-8088, Japan

Received October 8, 1998 Accepted February 19, 1999

ABSTRACT—To clarify how vitamin K_2 prevents bone loss in vivo, it was given to ovariectomized 20-week-old rats for 2 weeks. Bone mineral density (BMD) in the whole femur and in 7 specific portions (F1 to F7 from the proximal to the distal end) was determined by dual-energy X-ray absorptiometry, and histomorphometry was also performed in proximal tibial metaphysis. Ovariectomy (OVX) resulted in significant decreases in the BMD in the whole femur and the F1, F2, F6 and F7 portions. Histomorphometrical analysis of the tibia showed that the bone volume / tissue volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N) were decreased, while trabecular separation (Tb.Sp) and osteoclast number / bone surface (Oc.N/BS) were increased by OVX. The parameters for bone formation were not changed by OVX. These data indicate that the bone loss within 2 weeks is due to the enhancement of bone resorption. Vitamin K_2 at 50 mg/kg inhibited the decrease in the BMD of the whole femur together with the F6 and F7 portions. Vitamin K_2 also inhibited the decrease in Tb.N and the increases in Tb.Sp, Oc.N/BS and osteoclast surface / bone surface (Oc.S/BS) caused by OVX. These results suggest that vitamin K_2 prevents bone loss through the inhibition of bone resorption and osteoclast formation in vivo.

Keywords: Vitamin K_2, Ovariectomy, Bone mineral density, Histomorphometry, Osteoclast number

Many studies suggesting a relationship between vitamin K and bone metabolism have been reported; e.g., the circulating level of vitamin K (1-4) or undercarboxylated osteocalcin (5, 6) is related to the risk of fractures in osteoporotic patients, and vitamin K supplementation in postmenopausal women (7) or vitamin K-depleted rats (8) induces a decrease in the urinary excretion of calcium and hydroxyproline.

Menatetrenone, vitamin K_2 with 4 isoprene units, has high γ-carboxylation activity in hypoprothrombinaemic rats (9). Therefore, we studied the effect of menatetrenone on bone metabolism and found that it prevents bone loss induced by ovariectomy (10) or prednisolone administration (11) in rats. Moreover, menatetrenone has a significant therapeutic effect on involutional osteoporosis (12). In vitro studies indicated that vitamin K_2 inhibits bone resorption in an organ culture system (13), inhibits osteoclast-like cell formation in bone marrow culture (14) and co-culture systems (15) and enhances human osteoblast-induced mineralization (16). However, it has not yet been demonstrated that these in vitro effects of vitamin K_2 actually contribute to bone metabolism in vivo. It is generally accepted that estrogen deficiency in rats induces an immediate increase in bone turnover, as evidenced by rapid increases in osteoclast number, resulting in a rapid loss of trabecular bone (17-20). Therefore, in this study, to clarify the inhibitory effect of vitamin K_2 on bone resorption in vivo, we measured bone mineral density in the femur and histomorphometrically compared bone resorption and bone formation in the proximal tibia metaphysis in rats treated with vitamin K_2 during the early period after ovariectomy.

MATERIALS AND METHODS

Experimental protocol

A group of 40 female Fischer rats, 10 weeks of age, were obtained from Clea Japan, Inc. (Tokyo) and acclimated for 10 weeks prior to the start of the experiment with a standard diet (CE-2; Clea Japan, Inc.). At 20 weeks of age, they were divided into 4 groups: sham, ovariectomy (OVX)-control, OVX-vitamin K_2 5 mg/kg

and OVX-vitamin K_2 50 mg/kg. Under pentobarbital anesthesia, rats in the 3 groups were bilaterally ovariectomized and the remainder were subjected to sham surgeries. All animals were fed a synthetic diet containing 0.5% calcium, 0.78% phosphorus and 240 IU/100 g vitamin D_3 (Clea Japan, Inc.). Vitamin K_2 (menatetrenone; Eisai Co., Ltd., Tokyo) was given as a dietary supplement for 2 weeks. The vitamin K_2 content of the diet in the OVX-vitamin K_2 5 and 50 mg/kg groups was 9.4 and 85.0 mg per 100 g diet, respectively, calculated by the mean body weight (178.8 g) and daily food intake/rat (10.1 g). In this study, food intake of all the rats was restricted to the same amount of food, 10.1 g per rat per day, to prevent the hyperphagia that occurs in ovariectomized animals. Distilled water was given ad libitum. They were kept in a room maintained at 24±2℃ with a 12-hr light-dark cycle. At days 11 and 5 before sacrifice, these rats were given 2 subcutaneous injections of tetracycline (Teramycin®; Pfizer, Tokyo) at a dose of 25 mg/kg in order to label the sites of active bone formation. The body weights of all animals were measured at the time of ovariectomy and at sacrifice. Two weeks after the ovariectomy, these rats were sacrificed under pentobarbital anesthesia, and the success of the ovariectomy was confirmed by the weights of the uterine horns. Then bilateral femora and tibiae were harvested.

Bone mass measurements

After the length of the left femur was measured, femoral bone mineral content (BMC), bone mineral density (BMD) and the area of the whole femur were measured by dual-energy X-ray absorptiometry (DXA, QDR-1000; Hologic Inc., Waltham, MA, USA) using special software developed for small animals, set to a high resolution mode. Both intra- and interassay coefficients of variation (CVs) for BMD were less than 1%. As the length of the femora in the 4 groups was almost the same, the scan image of the femur was divided into 7 portions of equal length (Fig. 1), and BMC and BMD in each portion were calculated using this software.

Preparation of specimens

The tibiae were removed and dissected from the adhering connective tissue and muscle before being cut 10 mm from the proximal end. The proximal portions of the right tibiae were fixed with 70% alcohol and stained using Villanueva bone stain (Maruto Instrument Co., Ltd., Tokyo) for 7 days. They were then dehydrated with sequential changes (70%, 95% and 100%) of an ethanol solution and embedded in methyl-methacrylate (Wako Pure Chemical Industries, Ltd., Osaka) without decalcification, and 8-μm-thick longitudinal sections were obtained with a Jung rotary microtome (Leica Instruments GmbH, Heidelberg, Germany). Some of these sections were then stained by Goldner's trichrome method for measurements of static parameters. Those sections without Goldner's stain were used for the measurement of dynamic parameters.

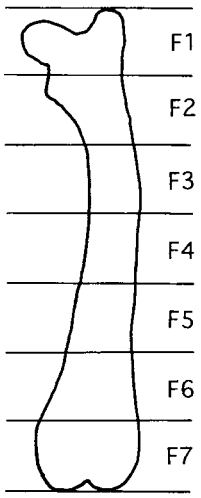

Fig. 1. A divided image of a femur by dual-energy X-ray absorptiometry (Hologic QDR-1000). After bone mineral content (BMC), bone mineral density (BMD) and the area of the whole femur were determined, the parameters of each of the 7 divided portions of equal length were calculated with special software.

The proximal portions of the left tibiae were fixed with 10% formalin in phosphate-buffered saline. They were dehydrated, embedded in glycolmethacrylate (2-hydroxyethyl methacrylate EM; TAAB Laboratories Equipment, Ltd., Berks, England) and longitudinally sectioned at the center to yield 8-μm frontal undecalcified sections. These sections were stained with tartrate-resistant acid phosphatase (TRAP) as a marker of osteoclast.

Histomorphometric analysis

Bone histomorphometry of the secondary spongiosa of the proximal metaphysis between 0.8 and 3.2 mm distal to the growth plate-epiphyseal junction (Fig. 2) was performed using an automated image analysis system as reported previously (21). The system consisted of an epifluorescent microscope (OPTIPHOTO-2; Nikon Co., Tokyo), a CCD color camera (HCC-3600; Flovel, Tokyo), a real time image analyzer (Luzex-F; Nireco Co., Tokyo), an autoscanner (MicroScaner; Sapporo Breweries Ltd., Tokyo) and a Macintosh computer.

Measurements and calculations-related static parameters were as follows: total metaphyseal area (TV, mm^2): metaphyseal area between 0.8 and 3.2 mm distal to the growth plate-epiphyseal junction without the cortices, trabecular bone area (BV, mm^2): total area of trabecular within TV, trabecular bone surface (BS, mm): length of

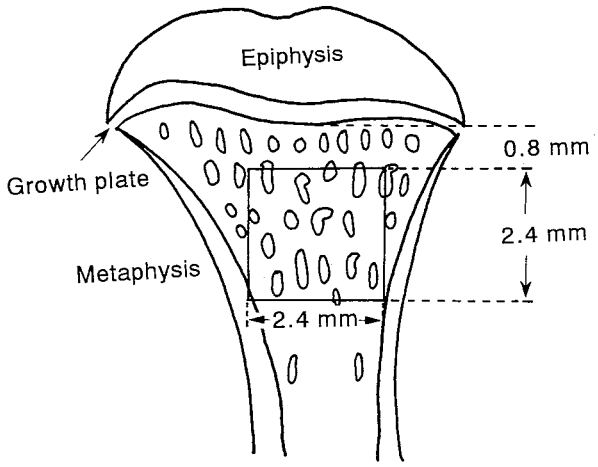

Fig. 2. Schematic representation of the sampling sites of the tibia employed for histomorphometric study. A 2.4 mm × 2.4 mm area of the proximal tibia metaphysis is shown.

the total perimeter of trabeculae, trabecular bone volume (BV/TV, %): BV/TV × 100, trabecular thickness (Tb.Th, μm): 2000/(BS/BV), trabecular number (Tb.N, #/mm): (BV/TV) × 10/Tb.Th, and trabecular separation (Tb.Sp, μm): Tb.Th × [(100/BV/TV) − 1].

Measurements and calculations related to dynamic parameters were as follows: single-labeled surface (sLS, mm): total length of trabecular surface labeled with one tetracycline label, double-labeled surface (dLS, mm): total length of trabecular surface labeled with two tetracycline labels, inter labeled thickness (IrLTh, μm): average distance between 2 tetracycline labels, mineral apposition rate (MAR, μm/day): IrLTh/label interval, and bone formation rate per bone surface (BFR/BS, $\mu m^3/\mu m^2$/day): (sLS/2 + dLS) × MAR/BS.

Measurements related to bone resorption were as follows: osteoclast number per bone surface (Oc.N/BS, #/mm): the number of TRAP-positive cells situated on the bone surface, and osteoclast surface per bone surface (Oc.S/BS, %): the length of the outline of TRAP-positive cells contacting the bone surface. The abbreviations for histomorphometric parameters are derived from the recommendation of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (22).

Statistical analyses

All data are expressed as the mean ± standard error of the mean (S.E.M.). Comparison of 2 groups (sham-operated vs OVX-control) was performed by Student's *t*-test. Comparison of OVX-control and vitamin K_2-treated groups was performed by Fisher's least significant different test.

RESULTS

BMC and BMD in femur

The BMC and BMD in the whole femur and the 7 divided portions are shown in Fig. 3, Table 1 and Table 2, respectively. The BMC in the whole femur in the OVX-control group tended to be lower than that in the sham group ($P < 0.1$), while that in the vitamin K_2 50 mg/kg group was significantly higher than that in the OVX-control group ($P < 0.05$). Ovariectomy resulted in a decrease in the BMC of the F2 and F6 portions, and the BMC in the F6 portion of the vitamin K_2 50 mg/kg group was significantly higher than that in the OVX-control group.

The BMD in the whole femur from the OVX-control group was significantly lower than in the sham group ($P < 0.01$), while in the vitamin K_2 50 mg/kg group, it was significantly higher than in the OVX-control group

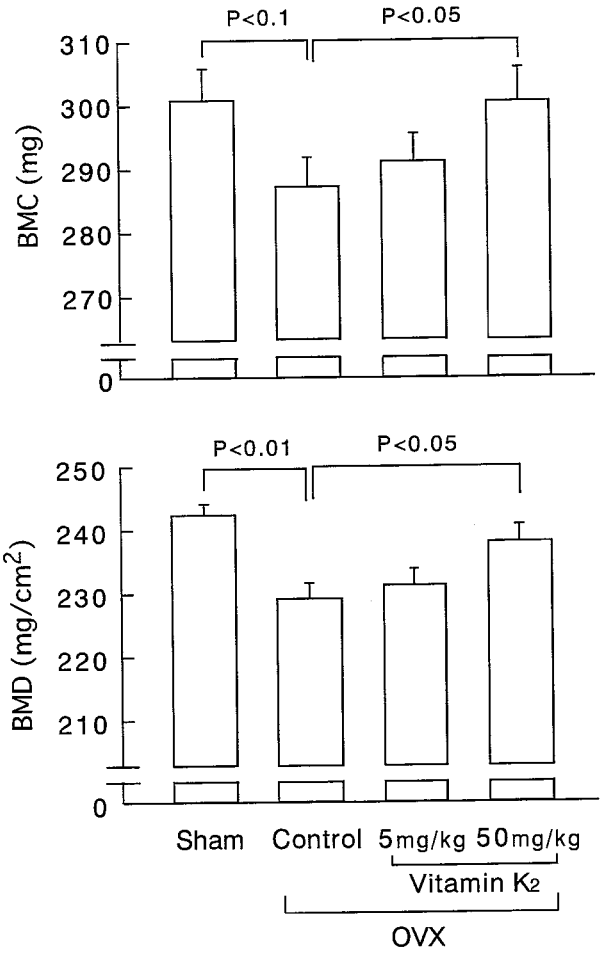

Fig. 3. Effects of vitamin K_2 on the bone mineral content (BMC) and bone mineral density (BMD) in the femur 2 weeks after ovariectomy (OVX). Vitamin K_2 (menatetrenone) was given as a dietary supplement. Each column and bar represents the mean ± S.E.M. (n = 10).

Table 1. Effect of vitamin K_2 on bone mineral content (BMC) in 7 portions of the femur 2 weeks after ovariectomy

	Dose (mg/kg)	BMC (mg)						
		F1	F2	F3	F4	F5	F6	F7
Sham		50.4±1.1	48.5±0.7	38.0±0.7	32.6±0.7	30.9±0.8	44.2±1.2	69.9±1.9
OVX								
Control		48.3±1.0	46.4±0.7*	37.2±0.8	32.1±0.7	31.2±0.7	38.5±1.2**	66.3±1.1
Vitamin K_2	5	48.5±0.8	46.5±0.8	38.3±0.7	33.2±0.5	31.0±0.5	38.7±1.4	67.9±1.4
	50	51.3±1.5	47.7±0.8	38.5±0.7	33.5±0.7	32.4±0.9	42.0±0.8†	69.0±2.0

Vitamin K_2 (menatetrenone) was given as a dietary supplement. A whole femur was divided into 7 equal portions, F1 to F7 from the proximal to distal end. Each value represents the mean±S.E.M. (n=10). *$P<0.05$, **$P<0.01$ vs Sham; †$P<0.05$ vs OVX-Control.

Table 2. Effect of vitamin K_2 on bone mineral density (BMD) in 7 portions of the femur 2 weeks after ovariectomy

	Dose (mg/kg)	BMD (mg/cm^2)						
		F1	F2	F3	F4	F5	F6	F7
Sham		228.9±1.9	249.5±3.2	226.2±2.7	220.1±2.5	206.9±2.3	232.8±4.9	303.9±4.3
OVX								
Control		218.4±3.6*	235.7±2.2**	219.9±4.4	219.1±3.0	207.3±2.5	203.8±5.4**	278.5±3.6**
Vitamin K_2	5	216.1±2.2	237.7±3.3	222.7±2.0	220.1±2.1	206.6±2.5	205.1±5.6	286.1±4.3
	50	222.1±3.0	241.1±3.3	229.1±3.6	227.0±3.1	214.4±3.5	220.0±3.3†	291.5±4.3†

Vitamin K_2 (menatetrenone) was given as a dietary supplement. A whole femur was divided into 7 equal portions, F1 to F7 from the proximal to distal end. Each value represents the mean±S.E.M. (n=10). *$P<0.05$, **$P<0.01$ vs Sham; †$P<0.05$ vs OVX-Control.

($P<0.05$, Fig. 3). The BMDs of the F1, F2, F6 and F7 portions in the OVX-control group was lower than those in the sham group, but those of the F3, F4 and F5 portions were not affected by OVX (Table 2). In the vitamin K_2 50 mg/kg group, the BMDs of the F6 and F7 portions were significantly higher than those in the OVX-control group (Table 2). In the vitamin K_2 5 mg/kg group, the BMC and BMD were slightly higher than those in the OVX-control group, but not significantly.

Bone histomorphometry

Figure 4 shows typical microphotographs of longitudinal sections of the proximal tibial metaphysis 2 weeks after ovariectomy. Trabecular bone in ovariectomized rats was markedly decreased compared to that in the sham group. In the vitamin K_2 50 mg/kg group, the decrease induced by ovariectomy was not observed. Table 3 summarizes the static histomorphometric parameters, BV/TV, Tb.Th, Tb.N and Tb.Sp, in the 4 groups. In the OVX-control group, BV/TV, Tb.Th and Tb.N were decreased, and Tb.Sp was increased compared to those in the sham group. Treatment with 50 mg/kg of vitamin K_2 significantly inhibited these changes in Tb.N and Tb.Sp induced by ovariectomy. These values in the 50 mg/kg of vitamin K_2 group were 96% and 110% of those in the sham group, respectively, and no significant difference was observed between these two groups (Student's *t*-test). Treatment with 5 mg/kg of vitamin K_2 also slightly inhibited the changes induced by ovariectomy.

Dynamic parameters and bone resorption parameters were determined in the sham, the OVX-control and the OVX-K_2 50 mg/kg groups since a significant effect of vitamin K_2 on bone loss was observed at the dose of 50 mg/kg, but not at 5 mg/kg. Neither ovariectomy nor treatment with vitamin K_2 affected the dynamic histomorphometric parameters, IrLTh, sLS/BS, dLS/BS, MAR and BFR/BS (Table 4). The parameters for bone resorption, Oc.N/BS and Oc.S/BS, in the TRAP-stained longitudinal sections of proximal tibia metaphysis were markedly higher in the OVX-control group than in the sham group ($P<0.01$), while those in the OVX-vitamin K_2 50 mg/kg group were significantly lower than in the OVX-control group (Fig. 5).

DISCUSSION

To understand the mechanism for how an agent prevents bone loss, it is important to clarify whether it inhibits bone resorption or enhances bone formation. In this study, to demonstrate that vitamin K_2 prevents bone

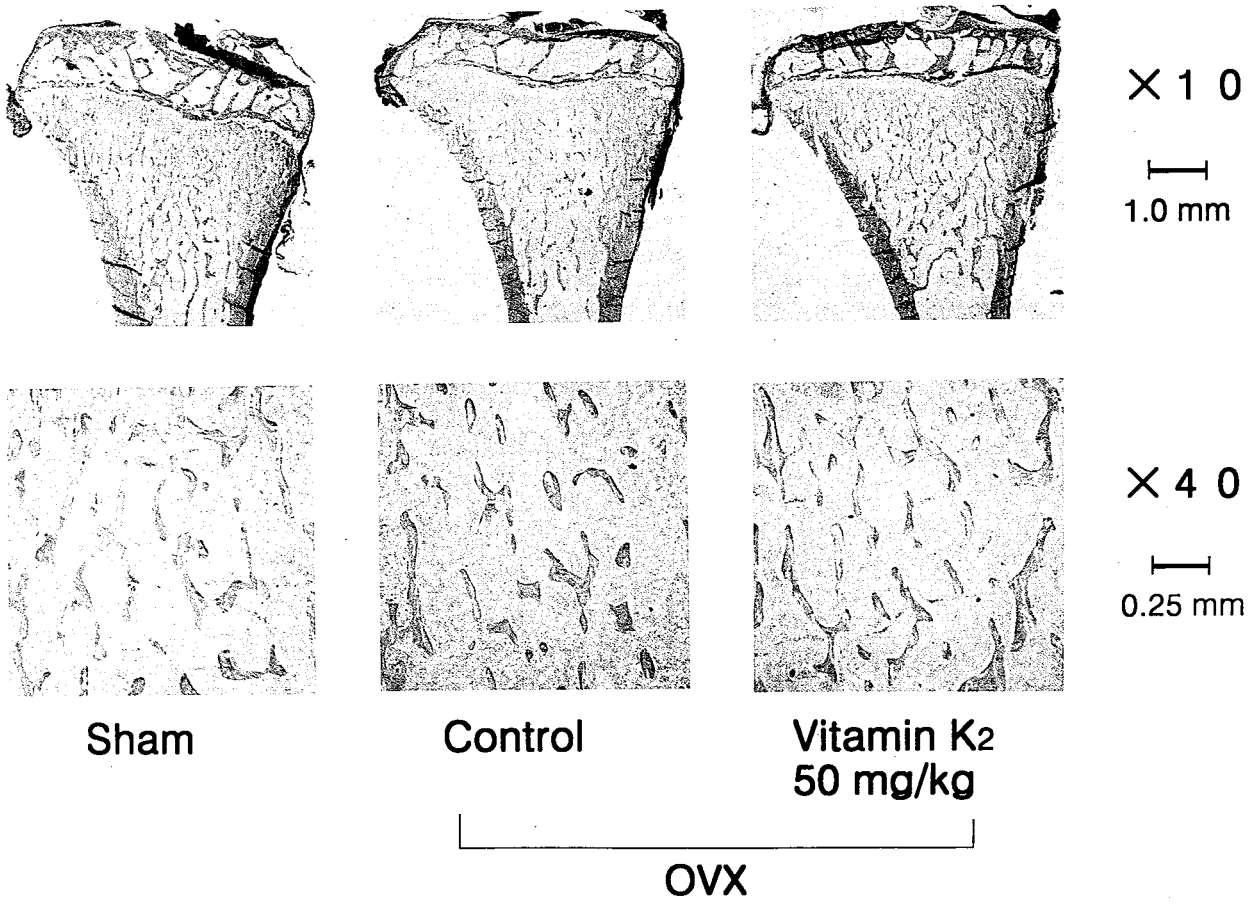

Fig. 4. Typical microphotographs of longitudinal sections of the proximal tibial metaphysis 2 weeks after ovariectomy. Tibias were cut 10 mm from the proximal end and fixed with 70% alcohol. After embedding in methylmethacrylate, they were sectioned longitudinally at 8-μm thickness and stained by the Villanueva Goldner's trichrome method.

Table 3. Effects of vitamin K_2 on bone histomorphological parameters in the longitudinal sections from the proximal tibia 2 weeks after ovariectomy

	Dose (mg/kg)	BV/TV (%)	Tb.Th (μm)	Tb.N (/mm)	Tb.Sp (μm)
Sham		21.7±1.3	64.4±1.9	3.35±0.15	239.3±14.4
OVX					
Control		15.1±0.9**	53.0±1.7**	2.83±0.12*	306.2±16.3**
Vitamin K_2	5	17.1±0.7	55.5±0.9	3.07±0.09	278.8±14.2
	50	17.7±1.1	54.8±1.7	3.20±0.12†	262.6±15.2†

Vitamin K_2 (menatetrenone) was given as a dietary supplement. Two weeks after ovariectomy, tibias were cut 10 mm from the proximal end and fixed with 70% alcohol. They were stained by Villanueva bone stain, dehydrated and embedded in methyl-methacrylate. Next, they were sectioned longitudinally at 8-μm thickness and stained by Goldner's trichrome method. As bone histomorphological parameters, the bone volume / tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were determined. Each value represents the mean±S.E.M. (n=10). *$P<0.05$, **$P<0.01$ vs Sham; $^{\dagger}P<0.05$ vs OVX-Control.

loss partly due to the inhibition of bone resorption in vivo, we used a bone loss model 2 weeks after ovariectomy in rats and evaluated the effect of vitamin K_2 by bone mass measurement and histomorphometric analysis.

Table 4. Effects of vitamin K_2 on the parameters of bone formation in the longitudinal section of the proximal tibia 2 weeks after ovariactomy

	Dose (mg/kg)	IrLTh (μm)	sLS/BS	dLS/BS	MAR (μm/day)	BFR/BS ($\mu m^3/\mu m^2$/day)
Sham		7.63±0.50	0.110±0.013	0.068±0.026	1.090±0.072	0.155±0.042
OVX						
Control		8.14±0.51	0.094±0.011	0.035±0.009	1.163±0.073	0.104±0.017
Vitamin K_2	50	7.13±0.41	0.088±0.017	0.048±0.009	1.018±0.058	0.094±0.011

Vitamin K_2 (menatetrenone) was given as a dietary supplement. Rats were subcutaneously treated with 25 mg/kg of tetracycline 11 and 5 days before sacrifice. Two weeks after ovariectomy, tibias were cut 10 mm from the proximal end and fixed with 70% alcohol. They were embedded in methyl-methacrylate and sectioned longitudinally at 8-μm thickness. As the parameters for bone formation, the inter labeled thickness (IrLTh), single labeled surface / bone surface (sLS/BS), double-labeled surface / bone surface (dLS/BS), mineral apposition rate (MAR) and bone formation rate (BFR/BS) were determined. Each value represents the mean±S.E.M. (n=9–10).

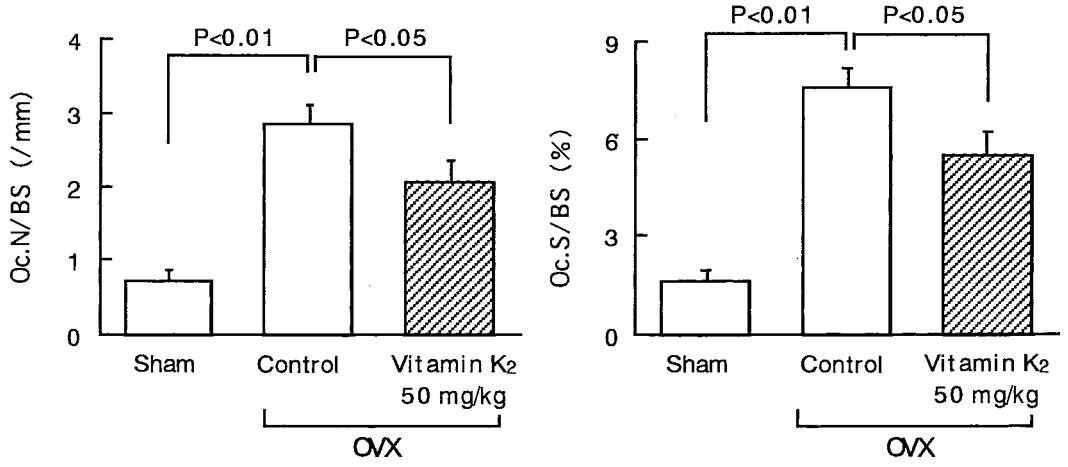

Fig. 5. Effects of vitamin K_2 on osteoclast number/bone surface (Oc.N/BS) and osteoclast surface/bone surface (Oc.S/BS) in longitudinal sections from the proximal tibia 2 weeks after ovariectomy. Vitamin K_2 (menatetrenone) was given as a dietary supplement. Tibias were cut 10 mm from the proximal end and fixed with 10% formalin in phosphate-buffered saline. After embedding in glycolmethacrylate, they were sectioned longitudinally at 8-μm thickness and stained with tartrate-resistant acid phosphatase stain. Each column and bar represent the mean±S.E.M. (n=10).

In the bone histomorphometry, Oc.S/BS and Oc.N/BS, as parameters of bone resorption, in the OVX-control group were 4.3 and 3.7 times as high as those in the sham group, respectively (Fig. 5). BV/TV and Tb.N in the OVX-control group were decreased to 69.6% and 84.5% of those in the sham group, respectively (Table 3). There were no significant differences in MAR and BFR/BS, parameters of bone formation, in the 2 groups (Table 4). Yamaura et al. (18) observed histomorphometrical changes in proximal tibial metaphyses during the early period after ovariectomy and indicated that OVX resulted in significant increases in Oc.S/BS and Oc.N/BS at the 3rd day, which were maintained thereafter, but dLS/BS and BFR/BS were not changed until 14 days after OVX and then increased at the 19th day. Dempster et al. (17) also observed histomorphometrical changes in the proximal tibial metaphysis every 5 days and reported that Oc.S/BS was increased on the 5th day, but BFR/BS was increased on the 15th day. These data indicate that the loss of trabecular bone during the early periods after ovariectomy, especially within 2 weeks, is due to the enhancement of bone resorption, and they agree with the histomorphometrical data in this study.

At 2 weeks after ovariectomy, BMC and BMD in the whole femur in the OVX-control group were both decreased to 95% of those in the sham group (Fig. 3). Moreover, the decreases in the F6 portion reached 87%, whereas in the F3, F4 and F5 portion, these changes were not observed (Tables 1 and 2). These data indicate that bone loss occurs early after ovariectomy, particularly in trabecular-bone-abundant portions. Geusens et al. (23) reported that OVX resulted in a decrease in the distal end

at 20% of the length of the femur, which contains more trabecular bone, but not in the middle portion, which contains cortical bone, after 3 months. A recent histomorphometrical study in femora showed that bone loss in the metaphysis was more rapid than in the epiphysis or diaphysis after ovariectomy (20). The F7 portion in this study corresponds to epiphysis, and the F6 portion to metaphysis. So the decrease in the BMC and BMD in the F6 portion after ovariectomy is thought to be caused by the increase in bone resorption.

Vitamin K_2 at 50 mg/kg significantly inhibited the decrease in BMC and BMD both in the whole femur and the F6 portion and the decrease in Tb.N and the increase in Tb.Sp in the tibial metaphysis. As mentioned above, bone loss 2 weeks after ovariectomy is due to increase in bone resorption, especially in the metaphysis, so vitamin K_2 is thought to cause an inhibitory effect on bone loss through the inhibition of bone resorption. Moreover, the inhibition of the increase in Oc.N/BS and Oc.S/BS caused by vitamin K_2 indicated that vitamin K_2 precisely inhibited bone resorption in the cell level.

We have already reported that vitamin K_2, 3×10^{-6} -3×10^{-5} M, inhibits bone resorption induced by PGE_2 and IL-1α in a mouse calvaria organ culture system dose-dependently (13). Moreover, osteoclast-like cell formation caused by $1,25(OH)_2D_3$ was strongly inhibited by 10^{-5} M of vitamin K_2 in mouse bone marrow cell culture (14) and co-culture systems (15). Kameda et al. (24) also reported that 10^{-6} M of vitamin K_2 inhibits the increase in the area of pit formation by isolated osteoclasts on dentine slices and induces apoptosis of osteoclasts. In these studies, it was shown that these effects after such a high dose of vitamin K_2 were not due to cell toxicity (13, 24). Sano et al. (25) reported that after once daily oral administration at a dose of 4 mg/kg for 10 days in female rats, the radioactivity concentration of vitamin K_2 in the bone marrow of the femur was higher than that in the plasma, reaching to 10^{-5} M. This level is consistent with the effective concentration of vitamin K_2 in in vitro studies. In this study, we did not determine the concentrations of vitamin K_2 in plasma or bone marrow, but the concentration of vitamin K_2 at 50 mg/kg administration seemed to reach the pharmacologically effective level in bone.

In conclusion, the data presented here show that the effect of vitamin K_2 on bone resorption reported in vitro actually contributes to its effect on bone metabolism in vivo.

Acknowledgments

We thank Ms. H. Yamauchi and Ms. I. Mitome for their technical assistance.

REFERENCES

1 Hart JP, Shearer MJ, Klenerman L, Catterall A, Reeve J, Sambrook PN, Dodds RA, Bitensky L and Chayen J: Electrochemical detection of depressed circulating levels of vitamin K_1 in osteoporosis. J Clin Endocrinol Metab **60**, 1268–1269 (1985)

2 Bitensky L, Hart JP, Catterall A, Hodges SJ, Pilkington MJ and Chayen J: Circulating vitamin K levels in patients with fractures. J Bone Joint Surg [Br] **70-B**, 663–664 (1988)

3 Hodges SJ, Pilkington MJ, Stamp TCB, Catterall A, Shearer MJ, Bitensky L and Chayen J: Depressed levels of circulating menaquinones in patients with osteoporotic fractures of the spine and femoral neck. Bone **12**, 387–389 (1991)

4 Hodges SJ, Akesson K, Vergnaud P, Obrant K and Delmas PD: Circulating levels of vitamin K_1 and K_2 decreased in elderly women with hip fracture. J Bone Miner Res **8**, 1241–1245 (1993)

5 Plantalech L, Guillaumont M, Vergnaud P, Leclercq M and Delmas PD: Impairment of gamma carboxylation of circulating osteocalcin (bone gla protein) in elderly women. J Bone Miner Res **6**, 1211–1216 (1991)

6 Szulc P, Chapuy MC, Meunier PJ and Delmas PD: Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. J Clin Invest **91**, 1769–1774 (1993)

7 Knapen MHJ, Hamulyák K and Vermeer C: The effect of vitamin K supplementation on circulating osteocalcin (bone gla protein) and urinary calcium excretion. Ann Intern Med **111**, 1001–1005 (1989)

8 Robert D, Jorgetti V, Lacour B, Leclerq M, Cournot-Witmer G, Ulmann A and Drüeke T: Hypercalciuria during experimental vitamin K deficiency in the rat. Calcif Tissue Int **37**, 143–147 (1985)

9 Akiyama Y, Hara K, Matsumoto A, Takahashi S and Tajima T: Comparison of intestinal absorption of vitamin K_2 (menaquinone) homologues and their effects on blood coagulation in rats with hypoprothrombinaemia. Biochem Pharmacol **49**, 1801–1807 (1995)

10 Akiyama Y, Hara K, Ohkawa I and Tajima T: Effects of menatetrenone on bone loss induced by ovariectomy in rats. Jpn J Pharmacol **62**, 145–153 (1993)

11 Hara K, Akiyama Y, Ohkawa I and Tajima T: Effects of menatetrenone on prednisolone-induced bone loss in rats. Bone **14**, 813–818 (1993)

12 Orimo H, Shiraki M, Fujita T, Onomura T, Inoue T and Kushida K: Clinical evaluation of menatetrenone in the treatment of involutional osteoporosis–A double-blind multicenter comparative study with 1α-hydroxyvitamin D_3. J Bone Miner Res **7**, Suppl **1**, S122 (1992)

13 Hara K, Akiyama Y, Tajima T and Shiraki M: Menatetrenone inhibits bone resorption partly through inhibition of PGE_2 synthesis in vitro. J Bone Miner Res **8**, 535–542 (1993)

14 Akiyama Y, Hara K, Tajima T, Murota S and Morita I: Effect of vitamin K_2 (menatetrenone) on osteoclast-like cell formation in mouse bone marrow cultures. Eur J Pharmacol **263**, 181–185 (1994)

15 Hara K, Akiyama Y, Nakamura T, Murota S and Morita I: The inhibitory effect of menatetrenone on bone resorption may be related to its side chain. Bone **16**, 179–184 (1995)

16 Koshihara Y, Hoshi K, Ishibashi H and Shiraki M: Vitamin K_2 promotes 1α,25$(OH)_2$ vitamin D_3-induced mineralization in

human periosteal osteoblasts. Calcif Tissue Int **59**, 466–473 (1996)

17 Dempster DW, Birchman R, Xu R, Lindsay R and Shen V: Temporal changes in cancellous bone structure of rats immediately after ovariectomy. Bone **16**, 157–161 (1995)

18 Yamaura M, Nakamura T, Tsurukami H, Hijioka A, Narusawa K, Ohnishi H, Ohta T and Hosoda K: Local bone turnover in the metaphysis of the proximal tibia and the lumbar vertebra during the early periods after ovariectomy in rats. Calcif Tissue Int **58**, 52–59 (1996)

19 Lane NE, Thompson JM, Haupt D, Kimmel DB, Modin G and Kinney JH: Acute changes in trabecular bone connectivity and osteoclast activity in the ovariectomized rat in vivo. J Bone Miner Res **13**, 229–236 (1998)

20 Baldock PAJ, Morris HA, Need AG, Moore RJ and Durbridge TC: Variation in the short-term changes in bone cell activity in three regions of the distal femur immediately following ovariectomy. J Bone Miner Res **13**, 1451–1457 (1998)

21 Kobayashi M, Akiyama Y, Tomiuga T, Hara K and Nakamura T: Automatic determination for bone morphometry using a computed image analyzer. J Jpn Orthop Assoc **69**, 1170–1181 (1995) (Abstr in English)

22 Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM and Recker RR: Bone histomorphometry: Standardization of nomenclature, symbols, and units. J Bone Miner Res **2**, 595–610 (1987)

23 Geusens P, Dequeker J, Nijs J and Bramm E: Effect of ovariectomy and prednisolone on bone mineral content in rats: Evaluation by single photon absorptiometry and radiogrammetry. Calcif Tissue Int **47**, 243–250 (1990)

24 Kameda T, Miyazawa K, Mori Y, Yuasa T, Shiokawa M, Nakamaru Y, Mano H, Hakeda Y, Kameda A and Kumegawa M: Vitamin K_2 inhibits osteoclastic bone resorption by inducing osteoclast apoptosis. Biochem Biophys Res Commun **220**, 515–519 (1996)

25 Sano Y, Tadano K, Kaneko K, Kikuchi K and Yuzuriha T: Distribution of menaquinone-4, a therapeutic agent for osteoporosis, in bone and other tissues of rats. J Nutr Sci Vitaminol **41**, 499–514 (1995)