Effects of Menatetrenone on Bone Loss Induced by Ovariectomy in Rats

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ABSTRACT—The effects of menatetrenone, a vitamin K₂ homologue, on bone loss induced by ovariectomy in rats were studied in 3 experiments. Menatetrenone was given as a dietary supplement. In experiment 1, at 2 weeks postovariectomy, menatetrenone (10 mg/kg/day given for 2 weeks) inhibited the decrease in bone density of the femoral metaphysis induced by the ovariectomy. In experiment 2, menatetrenone (3 or 30 mg/kg/day given for 6 months) inhibited the decrease in bone strength of the femur and the decrease in calcium and hydroxyproline content of the femoral diaphysis at 6 months postovariectomy. In experiment 3, menatetrenone treatment, at 30 or 100 mg/kg/day for 6 months, protected against the decrease in bone strength and calcium and hydroxyproline content in the bone loss model induced by ovariectomy and calcium-deficient diet. These findings suggest that menatetrenone protects against the bone loss induced by ovariectomy.

Keywords: Menatetrenone, Ovariectomy, Bone loss

Since Bouckaert and Said first reported the effect of vitamin K on fracture healing in 1960 (1), several studies have suggested that this vitamin affects bone metabolism. Hall et al. reported that some infants whose mothers had received oral anticoagulant therapy during pregnancy had hypoplasia of the nasal bone (2). Hart et al. demonstrated that circulating levels of vitamin K₁ in osteoporotic patients with fractures were significantly lower than those in age-matched control subjects (3). It is well-known that vitamin K is essential for the γ-carboxylation of osteocalcin (4), a non-collagenous protein present in bone (5, 6). Although the role of osteocalcin in bone metabolism remains obscure, it is possible that vitamin K may play a role in bone metabolism via this substance.

Two types of vitamin K occur in nature: vitamin K₁ (phyloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone), which is derived from plants, and vitamin K₂ (menaquinones), a series of vitamers with multi-isoprene units at the 3-position. In the present studies, we assessed the effects of menatetrenone (2-methyl-3-all-trans-tetraprenyl-1,4-naphthoquinone), a vitamin K₂ with four isoprene units, on bone loss induced by ovariectomy in rats in three different experiments: In experiment 1, we examined its effects on early metaphysial bone loss in the femur; in experiment 2, we studied its effects on the development of bone loss induced by ovariectomy over a 6-month period; and in experiment 3, we studied its curative effects over 6 months on bone loss induced by ovariectomy and a calcium-deficient diet.

MATERIALS AND METHODS

Animals
For experiment 1, we used 20-week-old female Fischer rats (Clea Japan, Inc., Tokyo); and for experiments 2 and 3, we used 40-week-old female Sprague-Dawley rats (Charles River Japan, Inc., Tokyo). The animals were housed in a room maintained at 26°C with a 12-hr light-dark cycle and were given free access to food and distilled water. All operations were performed under pentobarbital anesthesia.

Experiment 1
Twenty rats underwent bilateral ovariectomy and were then divided into two groups: control and a menatetrenone-treated group. Ten rats underwent a sham operation. All rats were fed a regular diet containing 0.8–1.2% calcium and 0.7–1.1% phosphorus (Clea Japan, Inc.). Rats in the menatetrenone-treated group were given food containing 20 mg menatetrenone per 100 g for 2 weeks from the day after operation. The mean dose of menatetrenone during this experiment was about 10
mg/kg/day, calculated from the mean body weight and the food intake. After 2 weeks, these rats were sacrificed, and the body weight, as well as bone length, dry weight, volume, and bone density of the femurs were measured.

Experiment 2

The effect of menatetrenone on the development of bone loss induced by ovariectomy was evaluated in 28 rats. Twenty-one rats underwent bilateral ovariectomy and were divided into 3 groups: control, a menatetrenone 3 mg/kg/day group, and a menatetrenone 30 mg/kg/day group. Sham operations were given to the remaining rats. All rats were fed a regular diet. Rats in the menatetrenone-treated groups were given food containing menatetrenone for 6 months from the day after the operation. Body weight and food intake were measured once a month. To examine whether the expected dose of menatetrenone had been taken, menatetrenone intake from the regular diet was calculated from these data. Thus, menatetrenone intake was adjusted to the previously decided amount throughout the experimental period. The mean daily doses in the 3 and 30 mg/kg/day groups over the 6-month period were 2.8±0.1 and 27.4 ±1.2 mg/kg/day, respectively. Body weight, plasma components, bone strength, and calcium and hydroxyproline content of the femoral diaphysis were measured at 6 months postovariectomy.

Experiment 3

Rats (n = 122) were divided into two groups: one group underwent bilateral ovariectomy (n = 89) and the other underwent the sham operation (n = 33). For 3 months after the day of operation, ovariectomized rats were given food containing 0.01% calcium and 0.67% phosphorus, and the sham-operated rats were given a regular diet. After 3 months, at the beginning of menatetrenone treatment, bone strength and calcium content of the femurs of 6 rats in each group were measured to determine the degree of bone loss induced by the ovariectomy and the low calcium diet. The values obtained at this stage are expressed as "pre" values. The remaining rats in the ovariectomy-0.01% calcium group were divided into 3 groups: the control and 30 and 100 mg/kg/day menatetrenone-treated groups. They were given food containing 0.17% calcium and 0.67% phosphorus. Menatetrenone was given as a dietary supplement. The amount of menatetrenone in the diet was determined the same way as in experiment 2. The rats in the sham group were fed a regular diet. This feeding regimen was carried out for 6 months. The mean daily doses of menatetrenone in the 30 and 100 mg/kg/day groups over the 6-month period, determined from the body weight and the food intake measured once a month, were 27.4 ±1.2 and 91.9±5.8 mg/kg/day, respectively. Therefore, the rats were almost constantly administered menatetrenone at the designated doses during the experimental period. At 1, 2, 3, and 6 months after treatment with menatetrenone, 6 or 7 rats in each group were sacrificed. Body weights, plasma components, bone strength, and calcium and hydroxyproline content of femurs were measured at each point.

Measurements

Blood: Blood samples were taken from the abdominal aorta while the animals were under pentobarbital anesthesia, and plasma was separated. Calcium and alkaline phosphatase activity in plasma were immediately measured with a calcium counter (CA-201, Hiranuma Co., Ltd., Tokyo) and a commercial kit (Alkaline-phospha K test-Wako, Wako Pure Chemical Industries, Ltd., Osaka), respectively.

Bone: In all experiments, both femurs were removed and dissected from the adhering connective tissue and muscle. In experiment 1, after the length and volume of the femurs were measured, they were rinsed in 3 changes of ethanol and 3 changes of acetone, dried overnight, and weighed (dry weight of whole femur). Then they were placed in water overnight and the epiphysis of the distal end was removed from each femur; another cut was then made 5 mm proximal to the first cut to separate the metaphysis. After the volume of each metaphysis was determined, they were dehydrated in 3 changes of ethanol and 3 changes of acetone, following which they were dried overnight and weighed. In experiment 2, the right femurs were used to determine bone strength, and the left femurs were cut 8 mm from the distal end, into 2 segments. The diaphysis, proximal segment, was homogenized in distilled water with a Physocotron (Nition Medical Supply Ltd., Chiba) and centrifuged at 3000 rpm for 5 min. The supernatant was discarded, and the bone pellet was rinsed in 5 changes of distilled water and 5 changes of acetone, dried overnight, and weighed. In experiment 3, the right femurs were used to determine bone strength, and the left whole femurs were homogenized, rinsed, and dried as described above. Aliquots of the bone powder were hydrolyzed with 6 N HCl at 130°C for 3 hr and the hydrolysate was used for the measurement of calcium and hydroxyproline content. Calcium and hydroxyproline were measured by the OCPC (o- cresolphthalein complexone) (Calcium C-test Wako, Wako Pure Chemical Industries, Ltd.) and Kivirikko methods (7), respectively.

Bone strength was measured by a 3-point bending test with a machine purchased from Natsume Seisakusyo Co., Ltd. (Tokyo). The values were expressed as the vertical power loaded on the center of the femoral bone when it was fractured. The volume of bone segment was meas-
ured according to Archimedes’ principle. First, the bone marrow cavity of bone segments was filled with agar. A glass beaker filled with water was weighed, and the bone was suspended with a thin wire and completely immersed in the water, taking care not to allow it to touch the bottom of the beaker; the container was then weighed again, the difference between these weights being the volume of the bone. Bone density was calculated as the ratio of the weight to the volume of bone.

Gla content in the femur was determined by Kuwada’s method (8) as follows: Bone powder, extracted with 10% formic acid, was lyophilized. It was then hydrolyzed with 2.5 N KOH and neutralized with HClO₄. The sample was pre-labeled with o-futaric acid and separated by HPLC (column: Nucleosil SSB packed 4.6 × 100 mm stainless steel column, elution buffer: 0.15 M citrate buffer (pH 5.8), flow rate: 1.5 ml/min, column temperature: 47°C). The column eluate was measured fluorometrically at excitation and emission wavelengths of 334 nm and 440 nm, respectively (F1000 Fluorescence Spectrophotometer, Hitachi Co., Ltd., Tokyo).

Statistical analyses
All data are expressed as the mean ± standard error (S.E.). Comparison of two groups (Sham-operated vs. ovariectomized control) was performed by Student’s t-test. Analysis of variance was performed in the ovariectomized control vs. menatetrenone-treated groups, and the significance of differences was determined by Dunnett’s multiple comparison test or the F-test.

RESULTS
Effects of menatetrenone on bone loss in femoral metaphysis two weeks after ovariectomy (experiment 1)
Two weeks after ovariectomy, there was a significant decrease in bone weight and bone density of both the whole femur and the femoral metaphysis, particularly of the metaphysis. Although the bone dry weight and bone density of the whole femur in the menatetrenone-treated group were higher than those in the ovariectomized control group, the difference was not significant. However, menatetrenone significantly inhibited the decrease in bone density of the femoral metaphysis (Table 1).

Effects of menatetrenone on the development of bone loss induced by ovariectomy (experiment 2)
Changes in body weight, calcium level and alkaline phosphatase activity in the plasma at 6 months after ovariectomy and treatment with 3 or 30 mg/kg/day menatetrenone are listed in Table 2. The body weight of ovariectomized rats was greater than that of sham-operated rats. Plasma alkaline phosphatase activity was significantly greater in ovariectomized rats than in sham-operated rats, and this increase was inhibited by menatetrenone at 30 mg/kg/day. There was no difference in plasma calcium level between the sham-operated group and the ovariectomized control group. Menatetrenone did not affect the plasma calcium level.

Ovariectomy resulted in a decrease in bone strength of the femur to 81% of that in sham-operated rats (Fig. 1). Bone strength in each of the menatetrenone-treated groups was higher than that in the ovariectomized control group, but the difference was not significant by Dunnett’s multiple comparison test. There was a significant difference between the control group and the combined menatetrenone-treated groups (3 and 30 mg/kg/day) using the analysis of variance F-test. The bone lengths of the femur in the sham, ovariectomized control, and menatetrenone at 3 and 30 mg/kg/day groups were 38.0±0.5, 37.3±0.5, 37.9±0.2, and 37.6±0.4 mm, respectively, and no significant differences among the

<table>
<thead>
<tr>
<th>Table 1. Length, volume, dry weight, and bone density of femurs of ovariectomized rats treated with menatetrenone for 2 weeks</th>
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<tr>
<td>n</td>
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<tr>
<td>Whole femur</td>
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<tr>
<td>Sham</td>
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<tr>
<td>O VX control</td>
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<tr>
<td>O VX menatetrenone</td>
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<tr>
<td>Metaphysis</td>
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<tr>
<td>Sham</td>
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<tr>
<td>O VX control</td>
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<tr>
<td>O VX menatetrenone</td>
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</table>

Rats were bilaterally ovariectomized (OVX) at 20 weeks of age. Menatetrenone was given as a dietary supplement, at a mean dose of about 10 mg/kg body weight/day. Each value represents the mean±S.E. †: P<0.05, ††: P<0.01, compared with the sham-operated group. ‡: P<0.10, ‡‡: P<0.05, compared with the OVX control group.
As shown in Fig. 2, the calcium content of the femoral diaphysis was lower in the ovariectomized control than in the sham-operated rats, but the difference was not significant, because the number of rats in the sham and ovariectomized control groups was small (3 each), and there was large variation in the values of the sham-operated group. Calcium content in the menatetrenone (30 mg/kg/day) group was significantly higher than that in the control group, and the decrease in hydroxyproline content was significantly inhibited by 3 and 30 mg/kg/day menatetrenone (Fig. 3). The dry weights of the diaphysis in sham, ovariectomized control, and menatetrenone at 3 and 30 mg/kg groups was 321±45, 269±6, 286±14, and 298±7 mg, respectively. Calcium and hydroxyproline content in terms of dry weight showed almost the same values in these groups. The calcium and hydroxyproline content of the diaphysis showed a good correlation with bone strength: r=0.883 and r=0.745, respectively.

Table 2. Body weight and plasma calcium and alkaline phosphatase in ovariectomized rats treated with menatetrenone for 6 months

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body weight (g)</th>
<th>Plasma calcium (mEq/l)</th>
<th>Plasma alkaline phosphatase (K-A-U)</th>
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<tbody>
<tr>
<td>Sham</td>
<td>7</td>
<td>453±25</td>
<td>4.98±0.08</td>
<td>13.0±1.1</td>
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<td>OVX control</td>
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<td>524±27</td>
<td>4.87±0.05</td>
<td>17.3±1.9</td>
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<td>4.84±0.03</td>
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<td>menatetrenone, 30 mg/kg</td>
<td>7</td>
<td>513±10</td>
<td>4.83±0.05</td>
<td>13.1±0.8</td>
</tr>
</tbody>
</table>

Rats were bilaterally ovariectomized (OVX) at 40 weeks of age. Menatetrenone was given as a dietary supplement for 6 months at a mean dose of about 3 or 30 mg/kg body weight/day. Each value represents the mean±S.E. †: P<0.05, compared with the sham-operated group. *: P<0.05, compared with the OVX control group.

Fig. 1. Effects of menatetrenone on bone strength of femurs 6 months after ovariectomy (OVX). Rats were bilaterally ovariectomized at 40 weeks of age. Menatetrenone was given as a dietary supplement. The figures in the columns indicate the percentage change from sham-operated group values. Each value represents the mean±S.E. for 7 rats. †: P<0.05, compared with the sham-operated group. *: P<0.05, compared with the OVX control group.

Fig. 2. Effects of menatetrenone on calcium content in the femoral diaphysis 6 months after ovariectomy (OVX). Conditions are described in Fig. 1. The number of rats in the sham-operated, OVX-control, and OVX-menatetrenone at 3 mg/kg and 30 mg/kg groups was 3, 3, 3, and 5, respectively. The figures in the columns indicate the percentage change from sham-operated group values. Each value represents the mean±S.E. †: P<0.05, compared with the OVX-control group.
Fig. 3. Effects of menatetrenone on hydroxyproline content in the femoral diaphysis 6 months after ovariectomy (OVX). Conditions are described in Fig. 1. The figures in the columns indicate the percentage change from sham-operated group values. Each value represents the mean±S.E. for 3 to 5 rats. *: P<0.05, **: P<0.01, compared with the OVX control group.

Fig. 4. Changes in the bone strength of femurs in ovariectomized (OVX) rats treated with menatetrenone for 6 months. Rats were bilaterally ovariectomized at 40 weeks of age and fed a 0.01% calcium diet for 3 months (pre). These rats were then fed a 0.17% calcium diet. Menatetrenone was given as a dietary supplement. Each value represents the mean ±S.E. for 6 to 7 rats. *: P<0.05, **: P<0.01, compared with the sham-operated group. **: P<0.01, compared with the OVX control group. X, sham-operated group; 0, OVX-control group; A, OVX-menatetrenone, 30 mg/kg; •, OVX-menatetrenone, 100 mg/kg.

Fig. 5. Changes in the calcium content of the femur in ovariectomized (OVX) rats treated with menatetrenone for 6 months. Conditions are described in Fig. 4. Each value represents the mean ±S.E. for 6 to 7 rats. *: P<0.05, **: P<0.01, compared with the sham-operated group. **: P<0.01, compared with the OVX-control group. X, sham-operated group; 0, OVX-control group; A, OVX-menatetrenone, 30 mg/kg; •, OVX-menatetrenone, 100 mg/kg.

**Effects of menatetrenone on bone loss induced by ovariectomy and 0.01% calcium diet (experiment 3)**

The changes occurring in bone strength over the 6-month period after treatment with menatetrenone are shown in Fig. 4. “Pre” indicates the point at 3 months after ovariectomy and 0.01% calcium diet feeding. The bone strength in sham-operated rats gradually decreased from month 2 to month 6. In ovariectomized control rats, after one month of 0.17% calcium feeding, there was a transient increase in bone strength, followed by a gradual decline from 1 to 6 months. In both the 30 and 100 mg/kg/day menatetrenone-treated groups, the changes in bone strength were the same as those in the ovariectomized control group during the first 3 months. However, at 6 months, bone strength was not decreased in the 30 mg/kg/day menatetrenone group compared to the ovariectomized control group, and the difference between these two groups was significant.

As shown in Fig. 5, the calcium content of the femur in ovariectomized control rats gradually decreased from 1 to 6 months, whereas the calcium content in the menatetrenone groups showed the same pattern of change as in the ovariectomized control group until 3 months. No decrease was observed in either of the menatetrenone-treated groups at 6 months, and the differences between each menatetrenone-treated group and the control group were significant. The hydroxyproline content in the femur changed in the same manner as the calcium content; and at 6 months, the values in both the 30 and 100 mg/kg/day groups were significantly greater than that in the control group (Fig. 6). The dry weight of the femurs in the sham, ovariectomized control, and menatetrenone at 30 and
Fig. 6. Changes in the hydroxyproline content of femurs in ovariectomized (OVX) rats treated with menatetrenone for 6 months. Conditions are described in Fig. 4. Each value represents the mean ± S.E. for 6 to 7 rats. †: P < 0.05, ‡: P < 0.01, compared with the sham-operated group. ×: P < 0.05 compared with the OVX-control group. ×, sham-operated group; ○, OVX-control group; ▲, OVX-menatetrenone, 30 mg/kg; ●, OVX-menatetrenone, 100 mg/kg.

Fig. 7. Effects of menatetrenone on the γ-carboxyglutamic acid (Gla) content of the femur in ovariectomized rats 6 months after treatment. Conditions are described in Fig. 4. Each value represents the mean ± S.E. for 6 to 7 rats. †: P < 0.05, compared with the sham-operated group. ×: P < 0.05, **: P < 0.01, compared with the OVX-control group.

Table 3. Body weight and plasma calcium and alkaline phosphatase in ovariectomized rats treated with menatetrenone for 6 months.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body weight (g)</th>
<th>Calcium (mEq/l)</th>
<th>Alkaline phosphatase (KA-U)</th>
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<tbody>
<tr>
<td>Sham</td>
<td>6</td>
<td>362 ± 25</td>
<td>4.82 ± 0.05</td>
<td>7.3 ± 0.8</td>
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<tr>
<td>OVX control</td>
<td>7</td>
<td>429 ± 16†</td>
<td>4.88 ± 0.06</td>
<td>8.6 ± 1.2</td>
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<td>menatetrenone, 30 mg/kg</td>
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<td>474 ± 34</td>
<td>4.78 ± 0.10</td>
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<td>menatetrenone, 100 mg/kg</td>
<td>6</td>
<td>390 ± 25</td>
<td>4.84 ± 0.07</td>
<td>10.0 ± 0.9</td>
</tr>
</tbody>
</table>

Rats were bilaterally ovariectomized at 40 weeks of age and fed a 0.01%-calcium diet for 3 months. They were then fed a 0.17%-calcium diet. Menatetrenone was given as a supplement to the 0.17%-calcium diet. Each value represents the mean ± S.E.. †: P < 0.05, compared with the sham-operated group.

100 mg/kg/day groups at 6 months was 591 ± 17, 536 ± 10, 583 ± 17, and 573 ± 12 mg, respectively. Calcium and hydroxyproline contents in terms of dry weight in these groups were almost the same.

The formic acid-soluble Gla content of the femur at 6 months was significantly lower in the ovariectomized control group than in the sham-operated group. In both of the menatetrenone-treated groups, the Gla content was significantly higher than that in the ovariectomized control group (Fig. 7).

Body weight and plasma calcium and alkaline phosphatase activity at 6 months are shown in Table 3. Animals which had been ovariectomized showed an increase of body weight compared to animals subjected to sham operation, but the menatetrenone-treated groups did not differ significantly from the control group. There was no change in plasma calcium levels after ovariectomy or menatetrenone treatment. Alkaline phosphatase activity in the ovariectomized control group was higher than that in the sham-operated group, and this activity tended to be higher in the menatetrenone groups than in the ovariectomized control group, although the differences were not significant.
DISCUSSION

Since the most common type of osteoporosis is that which develops postmenopausally, ovariectomized rats are widely used as an experimental model of bone loss (9–11). We used this model to assess the effects of menatetrenone on bone loss. Kimmel and Wronski reported that bone loss in ovariectomized rats develops differently in the metaphysis and diaphysis of the bone: in 14-week-old rats, bone mineral content in the distal end of the femur decreased after 5 weeks, whereas that in the diaphysis did not decrease over a long period (12). It has also been pointed out, by Jee et al., that old rats may become more amenable than young rats to cortical bone remodeling and possibly to cortical bone loss following ovariectomy (13). Therefore, to assess the effect of menatetrenone on metaphyseal and diaphyseal bone loss, we performed a short-term experiment in younger rats (Exp. 1) and long-term experiments in aged rats (Exp. 2 and 3).

As parameters of metaphyseal bone loss, we measured dry weight and bone density (Exp. 1). The bone density of the femur at 2 weeks after ovariectomy decreased to 94% and 85% of that in sham-operated rats in the whole femur and metaphysis, respectively, which was larger than the decrease in bone dry weight. These findings suggest that the bone density is a more sensitive parameter than bone dry weight to observe bone loss early after ovariectomy. Moreover, the bone loss in the metaphysis was more marked than that in the whole femur, a finding which is consistent with that of Kimmel and Wronski (12). Menatetrenone significantly inhibited the decrease in bone density of the metaphysis. To assess the effects of long-term menatetrenone administration on bone loss, we examined the changes in femoral diaphysis (Exp. 2 and 3).

In experiment 2, the development of bone loss in the diaphysis and the effects of menatetrenone on this loss were assessed at 6 months postovariectomy. The bone strength of the femur of ovariectomized rats was significantly lower than that in sham-operated rats at 6 months postovariectomy (Fig. 1). Since bone strength is measured at the center of the femur, it may reflect the breaking properties of the diaphysis. Menatetrenone significantly inhibited the decreases in bone strength and calcium and hydroxyproline contents in the diaphyseal bone, but did not affect the bone length. These findings suggest that this agent inhibits the loss of cortical bone.

It has been reported that feeding a calcium-deficient diet to ovariectomized rats accelerates bone loss (14, 15). Accordingly, a calcium-deficient diet (0.01% calcium) was fed to ovariectomized rats for 3 months, in experiment 3. After 3 months, the bone strength and calcium content of the femur in ovariectomized rats were significantly decreased, to 83% (P<0.05) and 86% (P<0.01), respectively, of the values in sham-operated rats. The dietary calcium content was then changed from 0.01% to 0.17%, because in a calcium-deficient state, the response to menatetrenone in this model will be small; however, in a normal calcium state (0.8%–1.2%), the ameliorative effect of calcium itself may be marked. Thus, a dietary calcium content of 0.17% was selected in order to observe the effects of menatetrenone. At one month after the diet was changed, the bone strength and calcium and hydroxyproline contents in the bone of ovariectomized groups had increased in comparison with the corresponding pre values; these increases may have been due to the influence of the increased dietary calcium content. In sham-operated rats fed a normal calcium diet throughout the experimental period, decreases in bone strength and decreases in the calcium and hydroxyproline content of the femur were observed from 2 to 6 months (from 15 to 19 months of age, Figs. 4–6). These changes may have been related to the aging of the rats. Menatetrenone inhibited the decreases in the bone strength and calcium and hydroxyproline contents of the femurs in ovariectomized rats.

The effects of menatetrenone on these 3 experimental osteoporosis models were examined using doses of 3 to 100 mg/kg/day. All the doses tested were effective in these models, but no clear dose-dependency could be demonstrated. The inhibitory effects of 30 and 100 mg/kg/day menatetrenone on bone loss were almost the same in experiment 3. The effective dose of menatetrenone in these models was thus considered to be 3 to 30 mg/kg/day.

Plasma alkaline phosphatase activity in ovariectomized control rats was higher than that in sham-operated rats, in experiment 2 (Table 2), a finding consistent with those of Kalu et al. (14) and Togari et al. (16). Plasma alkaline phosphatase activity is generally regarded as a biochemical marker for monitoring bone formation. The present results indicate that active bone formation and resorption occurred concurrently in ovariectomized rats. The low level of alkaline phosphatase activity shown in experiment 3 compared with that in experiment 2 may have been due to the difference in ages at the time of sacrifice. Alkaline phosphatase activity in the menatetrenone groups in experiment 2 was lower than that in the ovariectomized group, whereas it was higher in experiment 3; however, we are unable to explain the reason for this inconsistency.

Osteocalcin is synthesized only in osteoblasts (17), and newly synthesized osteocalcin releases into the circulation (18); thus, osteocalcin serum levels are regarded as a major biochemical marker of bone formation (19, 20).
Bone contains two Gla-proteins, osteocalcin and matrix Gla protein; the latter cannot be extracted in formic acid. In Experiment 3 we measured formic acid-extractable Gla in bone as a marker of osteocalcin. Menatetrenone increased the Gla content of bone, suggesting that this agent may affect osteoblast activity. Recently, Koshihara et al. reported that menatetrenone not only enhances mineralization but also increases the amount of osteocalcin produced by human osteoblasts in culture (21), finding which provides further evidence that menatetrenone affects osteoblast function.

It has been reported that the plasma parathyroid hormone (PTH) level and the sensitivity of bone to PTH is increased in ovariectomized rats compared to controls (22, 23) and that interleukin-1 (IL-1) production in peripheral blood monocytes and spleen macrophages obtained from postmenopausal women (24, 25) and from ovariectomized rats (26) is increased. Thus, PTH and IL-1 are both considered to play an important role in the excessive bone loss that often occurs during the postmenopausal period. Recently, we reported that menatetrenone dose-dependently inhibited the bone resorption induced by IL-1 and PTH in a mouse calvaria culture system (27), a result which supports the in vivo effects of menatetrenone demonstrated in the present study.

The inhibitory effect of menatetrenone on bone loss was also shown in a recent study, by Orimo et al., who assessed the therapeutic effects of menatetrenone on involu-tional osteoporosis; they showed that menatetrenone administered orally for 6–12 months significantly increased the bone mineral density of cortical bone (28).

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