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Time-Dependent Effects of Vitamin K2 (Menatetrenone) on Bone Metabolism in Postmenopausal Women

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Abstract. Vitamin K is known to mediate carboxylation of glutamyl residues of osteocalcin. We evaluated the effects of vitamin K2 (menatetrenone) treatment (45 mg/day) for 48 weeks on the markers of bone formation and resorption, bone mineral density (BMD), and the incidence of vertebral fractures in 34 Japanese postmenopausal women (aged 48–82 years). Serum levels of alkaline phosphatase (ALP) increased gradually and became significant at 48 weeks after menatetrenone treatment, while urinary excretion of deoxypyridinoline (DPD) decreased transiently but significantly at 4 weeks. Serum levels of both intact osteocalcin (OC) and carboxylated OC (Gla-OC) increased rapidly and significantly within 4 weeks and sustained their high values up to 48 weeks after the treatment, while those of undercarboxylated OC (Glu-OC) decreased reciprocally. These results can be interpreted to suggest that Glu-OC was converted to Gla-OC *in vivo*. On the other hand, lumbar BMD values showed no significant change and only one subject with a previous vertebral fracture had one newly occurring vertebral fracture. These results indicate that menatetrenone treatment of postmenopausal women constantly elevates bone formation markers as well as converts Glu-OC to Gla-OC. Thus, vitamin K2 treatment may promote bone formation, at least as measured biochemically in these subjects.

Key words: Vitamin K2, Postmenopausal women, Bone formation, Osteocalcin, Carboxylation

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OSTEOCALCIN (OC) is a bone-specific protein consisting of 49 amino acids and is one of the most abundant noncollagenous proteins in bone matrix. Since OC is synthesized mainly in osteoblasts and then released into the circulation, serum OC concentration is used as a bone formation marker [1, 2]. Moreover, OC contains three residues of γ-carboxyglutamic acid (Gla) at positions 17, 21, 24 of the amino acid sequence, which have a high specificity for calcium ions in hydroxyapatite and regulate the growth of these crystals [3–5]. *In vivo* studies using OC knockout mice and clinical cases with congenital malformations caused by the administration of warfarin during pregnancy suggest that Gla-containing OC is essential for the promotion of normal calcification of bone [6–10].

Vitamin K is a cofactor of γ-carboxylase that mediates the conversion of undercarboxylated OC (Glu-OC) to carboxylated OC (Gla-OC) by transforming glutamyl (Glu) residues of OC to Gla. Two types of vitamin K, vitamin K1 and vitamin K2, exist in nature. Vitamin K1 is a single compound, while vitamin K2 is a series of vitamers with multi-isoprene units (1 to 14) at the 3-position of the naphthoquinone. Recent *in vivo* and *in vitro* studies showed that both vitamin K1 and K2 not only activate blood coagulation [11–13], but also play important roles in the regulation of bone metabolism as a cofactor of γ-carboxylase. In particular, menatetrenone (MK-4) with 4 isoprene units has been reported to promote 1,25(OH)2 vitamin D3-induced mineralization of osteoblastic cells and to modulate the proliferation of the cells *in vitro* [14, 15]. Menatetrenone has also

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been reported to inhibit bone resorption by inhibiting osteoclast-like cell formation and by inducing osteoclast apoptosis *in vitro* [16–19]. The latter effect is based on the specific side chain of menatetrenone and is not observed in vitamin K1 or other vitamin K2 analogues. Moreover, *in vivo* experiments using rats showed that menatetrenone prevented bone loss induced by either ovariectomy or prednisolone treatment [20, 21]. However, the few clinical studies have investigated serum levels of bone formation markers as function of time, the various forms of OC such as intact OC, Gla-OC and Glu-OC, or the bone resorption markers during the administration of menatetrenone in humans. In this study, we examined these aspects in Japanese postmenopausal women who underwent menatetrenone treatment for 48 weeks. We also assessed changes in bone mineral density (BMD) at the lumbar spine and the incidence of vertebral fractures in the subjects during the treatment.

Subjects and Methods

Subjects

Subjects were 34 Japanese postmenopausal women aged 48–82 years (mean 65 yrs), who visited our outpatient clinic for a survey of osteoporosis. Ethics approval for this study was granted by our Institutional Review Board. The subjects gave their informed consent for monitoring biochemical parameters, BMD values, and fracture incidence. None of the subjects had diabetes mellitus, thyroid disorder or metabolic bone diseases, or was taking drugs or hormones that influence bone metabolism. Each participant was assessed to be in good health without any acute or chronic health problems that would make it inadvisable to receive menatetrenone. None of the subjects complained of any symptoms of menatetrenone side effects, such as epigastrial discomfort, nausea, and diarrhea, and thus all subjects completed this protocol.

Study design

The study was carried out in open conditions over 48 weeks. The patients were treated orally with 45 mg/day menatetrenone (Gla-kay: Eisai Co. Ltd., Tokyo, Japan). Adverse events were checked every 4 weeks. Biochemical examinations were performed just before and 4, 12, 24, and 48 weeks after the start of the treatment, and BMD measurements were performed just before and 12, 24, and 48 weeks after the start of the treatment. Lateral X-rays of the thoracic and lumbar spine were taken just before and 48 weeks after the start of the treatment.

Radiography

Vertebral fractures were assessed by lateral thoracic and lumbar spine radiographs and were defined using ratios of vertebral heights. Prevalent wedge fractures were defined by anterior heights more than 25% below posterior heights. Crush fractures were defined by midvertebral heights that were more than 20% below anterior or posterior heights. Ten subjects had total 16 compression vertebral fractures.

BMD measurements

Bone mineral density (BMD) at the lumbar spine (L2–4) was measured by dual-energy X-ray absorptiometry (DXA) (QDR 2000, Hologic Inc., MA, USA). The same operator tested all the woman during the study to eliminate operator discrepancies. The coefficients of variation (precision) of the measurements was 0.9%. Z-score means the deviation from the normal age- and sex-matched mean in standard deviations. Normative data were obtained from a population-specific database for Japanese women. A quality assurance test was carried out every day and the quality remained stable.

Biochemical measurements

Serum was promptly separated in the morning and stored at $-20°C$ until assay. Routine serum and urine chemistry determinations were carried out by standard automated techniques. Serum intact OC was assayed by an immunoradiometric assay (IRMA) using a tracer anti-OC (12–33) antibody and a solid-phase anti-OC (30–49) antibody with synthetic human OC (1–49) as a standard [22], which is expected to measure the major portion of OC that are produced during bone formation process [23]. The intra- and inter-assay variations were 4.6% and 6.3%, respectively, and the sensitivity of the assay

was 0.1 ng/ml. Serum levels of Gla-OC and Glu-OC were assayed by Gla-OC and Glu-OC EIA (Enzyme Immunoassay) Kits, respectively (Takara Shuzo Co., Shiga, Japan) [24]. Gla-OC EIA was performed with an antibody-POD conjugate [monoclonal antibody against residue (4–9) of OC] and with an antibody-coated microtiterplate against γ-carboxyglutamic acid at position 17 of bovine OC. The intra- and inter-assay variations were 6.6% and 14.9%, and the sensitivity of the assay was 0.2 ng/ml. Glu-OC EIA was performed with an antibody-POD conjugate [monoclonal antibody against residues (21–31) of OC] and with antibody-coated microtiterplate against glutamic acid 21 and 24 of human OC. The intra- and inter-assay variations were 7.3% and 8.5%, respectively, and the sensitivity of the assay was 0.5 ng/ml. Reactivity of the monoclonal antibody of this EIA system raised to bovine OC was 100% to Glu 17, 21, 24 position of OC and was 6% to Gla 17, 21, 24. Urinary total deoxypyridinoline (DPD) was quantified by HPLC using a fluorescence detector, as previously reported [25]. In brief, after urine samples were hydrolysed with HCL, they were ultrafiltrated and applied to CF1 cellulose columns with distilled water, and pidinium cross-links were separated by reversed-phase HPLC and detected by fluorophotometry. The intra- and inter-assay variations for total DPD were 7.5 and 10.1%, respectively. The sensitivity of the assay was 1.66 nmol/l. Each measurement was carried out individually and not at the same time.

Statistical analysis

Data were expressed as the mean ± SD. Wilcoxon signed rank test was used to determine whether menatetrenone treatment caused a significant change in the parameter measured, compared to pretreatment values. P-values less than 0.05 were considered significant.

Results

The baseline data in 34 postmenopausal women before menatetrenone treatment are summarized in Table 1. The levels of bone formation markers (intact OC and ALP) were within the normal ranges, while the level of DPD, a bone resorption marker, was slightly elevated. These data are compatible with those found in postmenopausal osteoporosis, in which bone resorption is known to exceed bone formation. The sum of serum levels of Gla-OC plus Glu-OC was not equal to that of intact OC. This observation can be explained because Gla OC with a γ-carboxylated amino acid at position 17 and glutamic acid at position 21, 24 is also counted as Glu-OC, and because several types of OC fragments as well as intact OC in the circulation are counted as either Gla-OC or Glu-OC due to the cross-reactivities of EIAs used in this study [23].

Table 1. Background data of subjects (n = 34)

Items	Units		Normal range
Age	years	65.4 ± 8.6	
YSM	years	16.4 ± 8.1	
Height	m	1.52 ± 1.05	
Weight	kg	50.0 ± 7.2	
Corrected Ca	mg/dl	9.11 ± 1.09	8.5–9.9
P	mg/dl	3.79 ± 1.16	2.5–4.5
ALP	IU/l	244.9 ± 54.6	100–303
Intact OC	ng/ml	7.65 ± 3.88	3.1–12.7
Gla-OC	ng/ml	9.67 ± 6.07	
Glu-OC	ng/ml	1.80 ± 1.35	
Pyr	pmol/μml/Cr	33.61 ± 9.32	17.7–41.9
DPD	pmol/μml/Cr	7.88 ± 4.83	2.2–6.1
BMD	g/cm^2	0.6546 ± 0.07	
T-score		−3.25 ± 0.65	
Z-score		−0.91 ± 0.58	

YSM: years since menopause

Table 2 shows time-dependent changes in biochemical markers during menatetrenone treatment. Serum calcium (Ca) levels increased slightly but significantly at 24 and 48 weeks after the treatment, while serum phosphorus (P) levels remained within normal ranges. Serum ALP levels increased gradually and became significant at 48 weeks. Intact OC levels elevated significantly and rapidly at 4 weeks and sustained their high values up to 48 weeks. In contrast, urinary DPD levels decreased transiently but significantly at 4 weeks and returned to normal ranges thereafter.

Fig. 1 shows time-dependent changes in serum levels of Gla-OC and Glu-OC during the menatetrenone treatment. Serum Gla-OC levels increased significantly and rapidly at 4 weeks and sustained their high values up to 48 weeks. In contrast, Glu-OC levels

Table 2. Time dependent % changes of biochemical and bone metabolic markers from the pretreatment values during menatetrenone treatment

	4w	12w	24w	48w
Ca	−0.1 ± 3.8	0.6 ± 3.7	2.5 ± 3.9*	2.1 ± 4.2*
P	1.4 ± 12.5	0.7 ± 10.1	4.3 ± 12.4	1.8 ± 7.2
ALP	2.1 ± 10.3	3.1 ± 11.1	4.6 ± 15.2	7.4 ± 18.7*
Intact OC	48.4 ± 122.2*	56.0 ± 97.8**	42.0 ± 74.7**	47.0 ± 70.8**
DPD	−9.0 ± 24.0*	2.6 ± 24.6	−5.2 ± 28.2	7.4 ± 33.2

*p<0.05 ** p<0.01

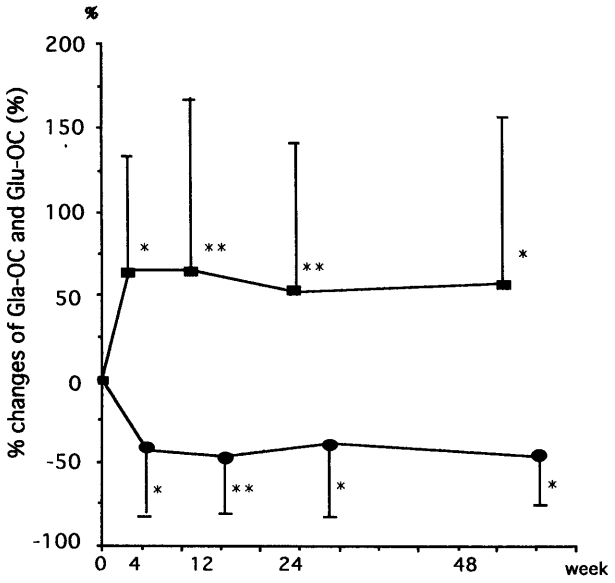

Fig. 1. The effect of menatetrenone on % change in Gla-OC and Glu-OC. Gla-OC (■) increased significantly and rapidly within 4 weeks and reached a plateau thereafter, while Glu-OC (●) decreased in a reciprocal manner.

decreased rapidly and significantly at 4 weeks and sustained their low values thereafter in a reciprocal fashion, suggesting that Glu-OC was converted to Gla-OC *in vivo*.

Fig. 2 shows time-dependent changes in the absolute value and Z score of lumbar BMD and % changes in the absolute value of lumbar BMD compared to the pretreatment value. These BMD values showed no significant changes during the treatment.

Table 3 shows the incidence of vertebral fractures before and after the menatetrenone treatment. Ten subjects had a total of 16 vertebral fractures before the treatment, and thus an average number of fractures per subject was 1.6. During the treatment, one new fracture occurred in one subject who also had one previous fracture. No new vertebral fractures occurred in the rest of the subjects regardless of fracture history.

Discussion

In the present study, menatetrenone treatment increased serum ALP levels gradually and significantly by 48 weeks and increased serum OC levels rapidly and significantly within 4 weeks. Two previous studies also showed that treatment of osteoporotic women with 45 mg/day menatetrenone increased serum levels of ALP and OC significantly at 4 and 24 weeks, respectively [26, 27], and Knapen *et al.* reported that a treatment with 1 mg/day vitamin K1, the other form of vitamin K, increased serum OC levels significantly in postmenopausal women [28]. Moreover, *in vitro* experiments showed that menatetrenone modulated the proliferation of osteoblastic cells with simultaneous increases in ALP activities and amounts of OC [14, 29]. Since both ALP and OC are biochemical markers of bone formation that are synthesized mainly in differentiating osteoblasts [30–32], this study together with the previous ones suggests that treatment of postmenopausal women with menatetrenone might promote bone formation, at least as judged by biochemical markers.

In contrast, the menatetrenone treatment in this study caused a transient but significant decrease in urinary excretion of DPD, a bone resorption marker. This result is compatible with *in vitro* findings that menatetrenone inhibited osteoclastic bone resorption by suppressing osteoclast-like cell formation and by inducing osteoclast apoptosis *in vitro* [16, 17, 19] and might reflect suppression of osteoclastic activity *in vitro*. Shiraki *et al.* have reported that menatetrenone treatment caused no significant decrease in urinary excretion of DPD at 12 or 24 months [27]. In

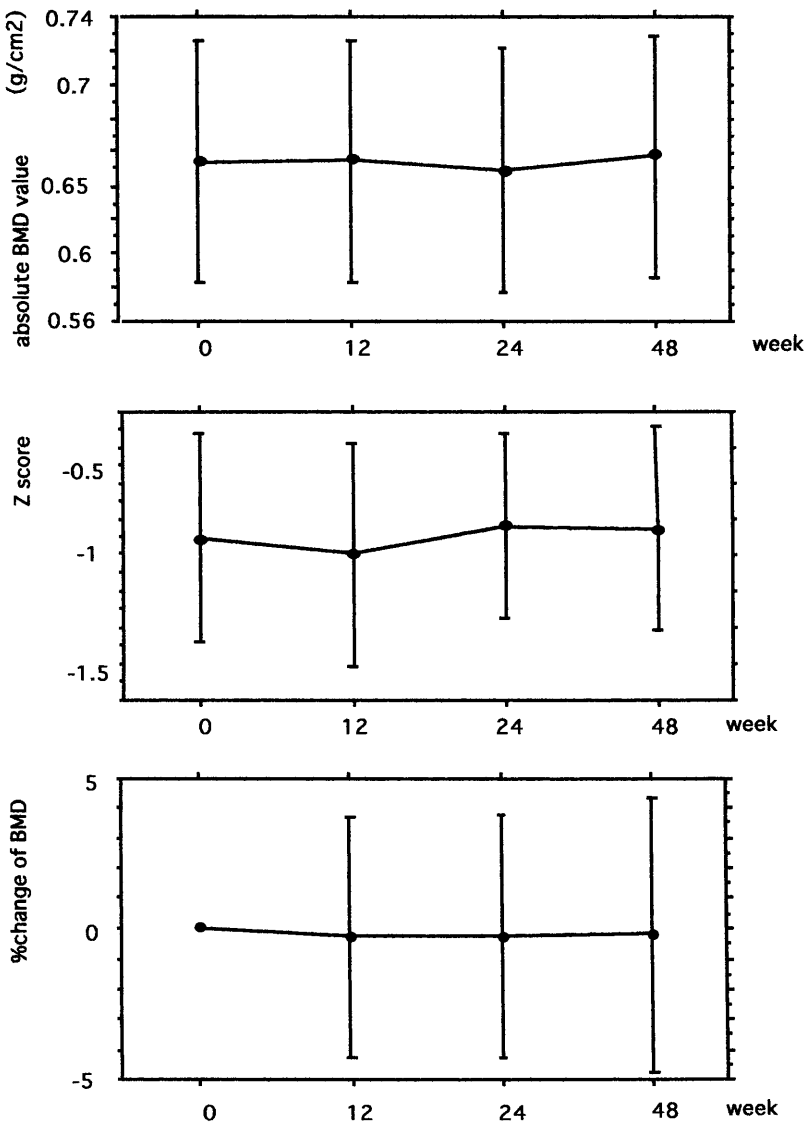

Fig. 2. Time courses of the absolute value and Z-score of lumbar BMD and of % change in the absolute value of BMD from pretreatment value during menatetrenone treatment. These parameters are plotted against weeks after starting menatetrenone treatment. Data are shown as mean ± SD.

Table 3. Vertebral fracture incidence in the subjects before and after menatetrenone treatment.

	Number of subjects with fractures	Total number of fractures	Average number of fractures per subject
Before	10	16	1.6
After	10	17	1.7

this study, significant decrease in urinary excretion of DPD was observed at 4 weeks, a much earlier time point, suggesting that menatetrenone may inhibit bone resorption soon after its administration but only transiently. In this study, serum Ca levels increased significantly at both 24 and 48 weeks after

menatetrenone treatment, although they were still within normal range. Menatetrenone has been reported to reduce urinary calcium excretion and to enhance calcium resorption in intestines [28, 34, 35], and these positive effects on calcium metabolism may explain this phenomenon.

The amount of Gla-OC in the blood has been assessed indirectly by either hydroxylapatite binding capacity or percentage carboxylation of serum immunoreactive OC [28, 36], or by the amount of Gla excreted in the urine [26]. In these indirect assays, both vitamin K1 and vitamin K2 were suggested to elevate serum Gla-OC levels *in vivo*. On the other hand, we measured serum levels of Glu-OC and Gla-OC directly and separately at several time points during menatetrenone treatment, and found that serum Gla-OC levels increased significantly and rapidly within 4 weeks after the treatment and reached a plateau thereafter, while those of Glu-OC decreased reciprocally, indicating that the treatment facilitated the conversion of Glu-OC to Gla-OC *in vivo*.

Subjects with low levels of serum vitamin K have been reported to be associated with low BMD [37] and with a higher incidence of femoral neck fractures [38–40]. An epidemiological study showed that low intakes of vitamin K1 may increase the risk of hip fracture in women [41]. Moreover, several studies showed that high Glu-OC levels have been associated with low BMD [42, 43] and with the higher incidence of femoral neck fractures [44–46]. These findings suggest the harmful effects of Glu-OC on both bone mass and fracture incidence. Thus, significant increases in serum levels of Gla-OC with reciprocal decreases in those of Glu-OC throughout the menatetrenone treatment in our subjects may be beneficial to the two parameters. Indeed, we observed that BMD did not decrease significantly and that only one new fracture occurred in one subject during the 48 week treatment. These findings are in accord with those of previous controlled studies showing that menatetrenone treatment prevented both decreases in BMD and the occurrence of new vertebral fractures [26, 27, 47, 48]. However, since this study was open, not placebo-controlled, and included only a small number of subjects, we should be cautious in the interpretation of the present data.

In conclusion, we examined the time course of bone markers as well as of lumbar BMD values and the incidence of vertebral fractures during treatment of postmenopausal women with menatetrenone, and found that several bone formation markers, including ALP, intact OC, and Gla-OC, increased significantly during the treatment. These findings suggest that menatetrenone has a stimulatory effect on bone formation as measured by biochemical markers.

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