Promotion of Bone Formation by Fermented Soybean (Natto) Intake in Premenopausal Women

Hironobu KATSUMA1, Seiji IDEGUCHI2, Masao FUKUNAGA3, Tatsushige FUKUNAGA4, Kiyofumi SAJOH5 and Shigeo SUNAMI1

Departments of 1Public Health, 2Health Care Medicine and 3Nuclear Medicine, Kawasaki Medical School, Kurashiki 701–0192, Japan
4Department of Forensic Medicine and Sciences, Mie University School of Medicine, Tsu 514–8507, Japan
5Department of Hygiene, Kanazawa University School of Medicine and Graduate School of Medical Sciences, Kanazawa 920–8640, Japan
(Received July 24, 2003)

Summary A therapeutic agent of vitamin K2 is approved for the treatment of osteoporosis in Japan. However, little is known about the efficacy of dietary intake of vitamin K2 for bone health. We compared the effects of various levels of fermented soybeans (Natto) intake, which contains plenty of vitamin K2, on bone stiffness and bone turnover markers in healthy premenopausal women. Seventy-three healthy premenopausal women were randomly divided into four groups matched for age and parity categories. Natto was supplied as follows: Group 1 (no intake), Group 2 (once per month), Group 3 (once per week) and Group 4 (three times per week). Subjects took Natto at a lunch for 1 y, and the stiffness index by quantitative ultrasound and bone turnover markers were assessed at baseline, 6 mo and 1 y.

There was no statistical difference in the stiffness index during the 1 y observation. However, bone specific alkaline phosphatase (BAP) in Group 4 was higher than that in Group 3 at 1 y and undercarboxylated osteocalcin (Glu) in Group 4 was significantly lower than those in Groups 1, 2 and 3 at 6 mo. Logistic regression analysis showed that the risk of reduction of bone formation markers declined to 0.07 in Group 4 based on that in Group 1.

In premenopausal women who had to keep the stiffness index as high as possible before menopause, Natto intake may have contributed to the promotion of bone formation.

Key Words premenopausal women, Natto intake, stiffness index, bone formation marker, vitamin K2

With the rapidly increasing population of the elderly in Japan, the incidence of osteoporosis and the number of elderly patients suffering from bone fractures are increasing (1). Although osteoporosis results in inadequate skeletal strength predisposing its sufferers to fracture, most osteoporotic patients are diagnosed after the occurrence of fractures since osteoporosis itself lacks specific symptoms. Moreover, U.S. Prevention Services Task Force (USPSTF), and 2002 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada do not recommend for routine osteoporosis screening in postmenopausal women who are younger than 60 or in women aged 60 to 64 who are not at increased risk for osteoporotic fractures (2, 3). Even though anti-resorptive therapies have been shown to reduce fracture risk and increase bone mineral density (BMD) (3), it is difficult to avoid the risk of fracture since it takes significant time to recover BMD except for treatment. In females, BMD reaches its maximal level in adolescence, and gradually decreases until menopause, after which it decreases dramatically (4). In order to prevent the incidence of involutional osteoporosis, it seems important not only to make a maximal level of BMD as high as possible in adolescence but also to maintain high BMD until menopause.

It is well known that nutritional and pharmacological factors are necessary to prevent age-related bone loss. Recently, it has been suggested that vitamin K2 may play an important role in both aspects. From the pharmacological perspective, one of vitamin K2 analog, menatetrenone or menaquinone-4 (MK-4), is approved as a therapeutic agent of osteoporosis in Japan. Since vitamin K2 activates osteocalcin through γ-carboxylation, its deficiency may increase the circulating level of undercarboxylated osteocalcin (Glu) and the risk of hip fracture (5). Moreover, subjects with osteoporotic patients treated with menatetrenone for 48 wk shows the significant increase in carboxylated osteocalcin (Glu) and decrease in Glu (6). Not only vitamin D3 administration alone but also the combined administration of vitamin D3 and vitamin K2 significantly increase BMD in postmenopausal women (7). On the other hand, from the nutritional perspective, the prospective cohort study including both premenopausal and postmenopausal women shows that dietary intake of vitamin K from vegetables, namely vitamin K1 intake, reduces the risk of femoral neck fracture (8). Soybeans fermented by Bacillus Natto (Natto), a traditional Japanese food, con-
tain more than 100 times more vitamin K₂ (mainly MK-7) than various cheeses (9). It has been reported that intake of Natto increases serum level of vitamin K₂, especially MK-7, and Gla in normal individuals (10). Higher incidence of hip fracture is found in women living in western Japan where people seldom eat Natto as opposed to eastern Japan where Natto is very popular (11, 12). Recently, it has been demonstrated that vitamin K₂ (MK-7) can directly stimulate calcification in the femoral metaphyseal tissues obtained from normal rats in vitro (13). Although both vitamin K₁ and MK-7 are reportedly converted to MK-4 (14), serum concentration of MK-4 is not measured (15). Since bioavailability from food and their divergent metabolism in vivo are yet unknown, the efficacy of continuous intake of neither vitamin K₂ nor vitamin K₁ in the development and maintenance of bone has not been fully clarified.

Since Natto is recognized to be a healthy food, it seems noteworthy to examine its efficacy for maintenance of bone health. Thus, we investigated the effects of various levels of Natto intake on bone stiffness and bone turnover markers in healthy premenopausal women with consideration of their lifestyle.

SUBJECTS AND METHODS

Subjects and lifestyle factors. One hundred and one healthy female workers at a local hospital in Japan were recruited with their written, informed consent. Twenty-eight workers were excluded because of dropping out, menopause, pregnancy, cardiovascular diseases, bilateral ovariectomy, use of anticoagulant therapy, or neglection of protocol, so that 73 premenopausal workers (mean age 33.5±10.0 y-old) were included in the present study. This study was aimed for the maintenance of bone health, not for treatment, hence all subjects were considered to be free from low bone mineral density. It is necessary to take into account for serum levels of vitamin K₂ due to the short half-life (approximately 2.5 d) (15). No statistical differences were observed in BMI, dietary intakes such as energy, protein, calcium and vitamin D, physical activity, and stiffness index among subjects divided by the frequencies of Natto intake prior to study.

None of the subjects had an unbalanced diet for vitamin K₁ rich food, e.g. green vegetables and fermented foods, indicating that there was no difference in vitamin K₁ intake among each group. The classic role of vitamin K₁ involves the synthesis of several blood coagulation factors (18, 19), and the maintenance of plasma prothrombin concentrations is the basis for the recommended dietary allowance of 1 μg/kg/d (20). Since subjects took vitamin K₁ from vegetables around recommended dietary allowance levels, the difference of vitamin K₂ intake could be assessed.

When stiffness index, bone turnover markers, and other health examinations were performed at baseline, 6 mo and 1 y, we also confirmed that the subjects were keeping to the protocol and examined dietary intake, physical activity and stature of body.

Stiffness index. The speed of sound (SOS; m/s) and the broadband ultrasound attenuation (BUA; dB/MHz) in the calcaneus were measured using an Achilles ultrasound bone densitometer (A-1000, Lunar Corporation, Madison, WI). SOS was recognized to reflect density and elastic properties of bone, and BUA was thought to relate to the structure and density of bone (21). The mathematical index, designated as ‘stiffness index’ was automatically calculated according to the formula: ‘stiffness index’ =0.67×BUA+0.28×SOS−420 (4, 22). Since the index was suitable for Caucasians, ‘% young adult’ was further calculated by dividing the stiffness index of each subject by the peak stiffness of normal Japanese females, which was 91.6 (4), to estimate bone mineral loss associated with aging.

Bone turnover markers. Second morning urine specimens were collected and blood samples were obtained when quantitative ultrasound (QUS) measurement was performed. Both serum and urine specimens were stored at −20°C until measurement. Not only bone spe-
specific alkaline phosphatase (BAP) but also Glu and Gla in serum were measured as bone formation markers. Glu and Gla were measured using the Glu-type and the Gla-type osteocalcin EIA kits (Takara Bio Inc., Kyoto, Japan). Bone resorption markers in urine were measured by creatinine, Ca (Ca/Cr) and type I collagen crosslinked N-telopeptides (NTx/Cr).

Health examinations. BMI, dietary intakes such as energy, protein, calcium and vitamin D, and physical activity at both 6 mo and 1 y did not change those at baseline, so that baseline data was used for statistical analysis. Menstrual cycles of subjects were not taken into account because regular health examinations were utilized as an occasional investigation. Changes in stiffness index and bone turnover markers (Δ6 mo and Δ1 y) were calculated.

Statistical analysis. One-way analysis of variance (ANOVA) with Fisher's PLSD as a post hoc test was performed to compare differences in lifestyle factors, stiffness index and bone turnover markers for each vitamin K2 intake group at baseline. Two-way ANOVA with Sheffe's test as a post hoc test was performed to compare the stiffness index and bone turnover markers for each vitamin K2 intake group and trend in each group. Risks in reduction of bone formation markers or increase of bone resorption markers were calculated using logistic regression analysis. These statistical analyses were calculated using Stat View (SAS Institute Inc., version 5.0).

RESULTS

Baseline characteristics

No difference was observed among the mean age of each group (Table 1). Although daily calcium intake of all groups was lower than the recommended intake in Japan, i.e., 600 mg/d, no statistical difference was observed among each group. There were no statistical differences in energy, protein and vitamin D intake, and physical activity. Mean values of BMI, calcium, vitamin D, energy and protein intakes, and physical activity of all subjects were 21.6 ± 2.7, 497.3 ± 217.3 mg/d, 177.8 ± 82.0 IU/d, 1,767.4 ± 343.5 kcal/d, 63.7 ± 17.8 g/d, and 8,465.1 ± 2,720.8 steps/d, respectively. Subjects took brightly colored vegetables constantly and their dietary intake was the same as Japanese standards as previously reported (23). Although the stiffness index of Group 1 was slightly higher than those of other groups, no statistical difference was observed. No statistical differences were observed in BAP, Glu, Gla, NTx/Cr and Ca/Cr.

Moreover, no statistical differences were observed in BMI, dietary intake such as energy, protein, calcium and vitamin D, and physical activity during the observation period.

Table 1. Baseline characteristics in each Natto intake group.

|                | Group 1 | Group 2 | Group 3 | Group 4 |
|----------------|---------|---------|---------|---------|
| n              | 18      | 21      | 16      | 18      |
| Age (yr)       | 33.6 ± 10.1 | 30.4 ± 9.8 | 34.8 ± 9.0 | 35.8 ± 11.0 |
| Weight (kg)    | 53.7 ± 11.0 | 53.7 ± 4.4 | 53.1 ± 8.2 | 53.4 ± 6.1 |
| BMI (kg/m²)    | 21.5 ± 3.2 | 22.1 ± 2.2 | 21.5 ± 3.1 | 21.4 ± 2.8 |
| Calcium intake (mg/d) | 511.9 ± 172.8 | 462.8 ± 161.0 | 456.8 ± 172.6 | 558.8 ± 325.7 |
| Vitamin D intake (IU/d) | 163.6 ± 62.2 | 178.4 ± 75.6 | 156.3 ± 60.9 | 210.7 ± 113.2 |
| Energy intake (kcal/d) | 1,736.0 ± 315.8 | 1,787.9 ± 303.0 | 1,740.1 ± 330.8 | 1,799.2 ± 438.1 |
| Protein intake (g/d) | 62.7 ± 18.2 | 63.2 ± 11.6 | 59.3 ± 12.0 | 69.4 ± 25.8 |
| Physical activity (step/d) | 8,294.9 ± 3,140.1 | 8,741.7 ± 2,679.1 | 8,056.0 ± 2,588.0 | 8,676.3 ± 2,609.5 |
| Stiffness index  | 95.1 ± 15.8 | 87.2 ± 10.6 | 85.5 ± 13.3 | 87.9 ± 12.3 |
| BAP (U/L)      | 60.8 ± 21.5 | 54.3 ± 16.6 | 51.9 ± 19.0 | 63.8 ± 19.8 |
| Glu (ng/mL)    | 3.1 ± 1.78 | 3.6 ± 2.11 | 3.12 ± 2.20 | 3.64 ± 1.89 |
| Gla (ng/mL)    | 1.91 ± 1.03 | 1.42 ± 0.99 | 1.95 ± 1.55 | 1.68 ± 1.46 |
| NTx/Cr (nm BCE/m² Cr) | 35.7 ± 14.0 | 45.8 ± 23.2 | 36.0 ± 15.3 | 46.7 ± 13.8 |
| Ca/Cr (g/g Cr) | 0.15 ± 0.09 | 0.13 ± 0.07 | 0.13 ± 0.07 | 0.12 ± 0.09 |

Natto intake group represents: Group 1: no intake, Group 2: 1/mo, Group 3: 1/wk, Group 4: 3/wk.

No statistical difference was observed in each group.

Table 2. Change of stiffness index in each group.

|                | Group 1 | Group 2 | Group 3 | Group 4 |
|----------------|---------|---------|---------|---------|
| Baseline      | 95.1 ± 15.8 | 87.2 ± 10.6 | 85.5 ± 13.3 | 87.9 ± 12.3 |
| 6 mo          | 90.7 ± 14.5 | 84.6 ± 10.4 | 80.4 ± 11.2 | 81.9 ± 11.3 |
| 1 y           | 94.5 ± 14.8 | 86.6 ± 11.4 | 85.6 ± 13.6 | 85.7 ± 12.0 |
| Δ6 mo         | -4.4 ± 6.2 | -2.6 ± 5.3 | -5.1 ± 7.7 | -6.0 ± 9.6 |
| Δ1 y          | -0.6 ± 7.4 | -0.6 ± 5.8 | 0.1 ± 9.1 | -2.3 ± 5.6 |

Δ6 mo: change of stiffness index at 6 mo (6 mo–baseline), Δ1 y: change of stiffness index at 1 y (1 y–baseline). No statistical difference was observed in each group.
Table 3. Changes of bone turnover markers in each group.

|                | Group 1            | Group 2            | Group 3            | Group 4            |
|----------------|--------------------|--------------------|--------------------|--------------------|
| BAP Baseline   | 60.8±21.5          | 54.3±16.6          | 51.9±19.0          | 63.8±19.8          |
| 6 mo           | 49.6±17.2          | 54.5±19.1          | 43.8±13.8          | 59.0±23.0          |
| 1 y            | 60.2±29.0          | 56.1±31.3          | 44.8±18.5          | 70.5±21.2*         |
| Δ6 mo          | -11.2±16.9         | 0.19±13.1          | -8.2±12.6          | -4.8±22.3          |
| Δ1 y           | -0.6±20.9          | 1.9±24.4           | -7.1±17.5          | 6.7±20.8           |
| Glu Baseline   | 3.11±1.78          | 3.63±2.11          | 3.12±2.20          | 3.64±1.89          |
| 6 mo           | 3.86±2.66          | 4.18±3.74          | 3.10±2.92          | 2.11±1.18          |
| 1 y            | 3.06±3.38          | 3.35±2.55          | 2.49±1.80          | 2.72±1.81          |
| Δ6 mo          | 0.76±1.50          | 0.54±2.40          | -0.02±1.19         | -1.53±2.06**       |
| Δ1 y           | -0.05±2.26         | -0.29±1.17         | -0.63±1.60         | -0.92±1.89         |
| Gla Baseline   | 1.91±1.03          | 1.42±0.99          | 1.95±1.55          | 1.68±1.46          |
| 6 mo           | 2.41±2.75          | 1.63±1.29          | 2.65±2.35          | 2.67±3.07          |
| 1 y            | 1.90±1.82          | 1.13±1.17          | 2.16±2.35          | 2.87±3.68          |
| Δ6 mo          | 0.50±2.24          | 0.21±0.79          | 0.71±1.74          | 0.99±1.94          |
| Δ1 y           | -0.01±1.43         | -0.29±0.73         | 0.22±1.68          | 1.18±2.88          |
| NTx/Cr Baseline| 35.7±14.0          | 45.8±23.2          | 36.0±15.3          | 46.7±13.8          |
| 6 mo           | 28.1±14.3          | 31.2±14.2          | 25.9±9.9           | 31.2±15.6          |
| 1 y            | 34.9±29.0          | 33.8±13.6          | 27.7±9.2           | 44.5±31.0          |
| Δ6 mo          | -7.6±16.0          | -14.6±19.1         | -10.1±11.5         | -15.5±14.1         |
| Δ1 y           | -2.7±24.4          | -12.0±16.7         | -11.7±19.1         | -2.2±30.4          |
| Ca/Cr Baseline | 0.15±0.09          | 0.13±0.07          | 0.13±0.07          | 0.12±0.09          |
| 6 mo           | 0.19±0.08          | 0.15±0.10          | 0.15±0.06          | 0.17±0.11          |
| 1 y            | 0.12±0.07          | 0.14±0.09          | 0.16±0.08          | 0.15±0.09          |
| Δ6 mo          | 0.04±0.11          | 0.02±0.13          | 0.02±0.08          | 0.06±0.10          |
| Δ1 y           | -0.03±0.06         | 0.01±0.12          | 0.02±0.08          | 0.03±0.08*         |

Δ6 mo and Δ1 y: See legend of Table 2.
Statistical difference in BAP was observed between Group 3 and Group 4 at 1 y (ANOVA, *p<0.05). Statistical differences in Glu between Group 4 and other groups at Δ6 mo (**p<0.01) and in Ca/Cr between Group 4 and Group 1 at Δ1 y (* p<0.05) were observed (Kruskal-Wallis test).

Change of stiffness index in each group

Although the stiffness index of Group 1 was slightly higher than those of other groups throughout the experimental period, no statistical difference was observed (Table 2). There were also no statistical differences at Δ6 mo and Δ1 y. To assess which step of bone turnover was preferentially affected, alteration of bone turnover markers was examined.

Changes of bone turnover markers in each group

In BAP, no statistical difference in each group was observed at both baseline and 6 mo (Table 3). However, BAP in Group 4 was significantly higher than that in Group 3 at 1 y; although no statistical difference was observed at Δ6 mo and Δ1 y. Changes in Glu at baseline, 6 mo and 1 y did not display significant alterations among groups, while Δ6 mo in Group 4 revealed a significant decrease in comparison to the other groups. Moreover, Glu concentration of Groups 3 and 4 slightly decreased compared to baseline, 6 mo and 1 y, whereas statistical significance was observed or not. Although Glu did not show significant alteration in each group, that at 6 mo and 1 y was higher than that at baseline in Groups 3 and 4. There were no significant differences in NTx/Cr. On the other hand, Ca/Cr in Group 4 was higher than that in Group 1 at Δ1 y reflecting that Natto was made from soybean.

Monovariate analysis seemed to be insufficient to estimate the effect of Natto intake as risk determinants so that logistic regression analysis was performed. Risk determinants in the reduction of bone formation marker or in the increase of bone resorption marker

Risk determinants of bone turnover markers were calculated using logistic regression analysis. Risk was adjusted by age, BMI, dietary intakes of protein, calcium and vitamin D, physical activity and vitamin K2 intake. BAP, Gla and Glu at 6 mo and 1 y were assessed as bone formation markers and NTx/Cr and Ca/Cr at 6 mo and 1 y were assessed as bone resorption markers. Risks in the reduction of bone formation markers were reduced to 0.07 in Group 4 based on Group 1 (Table 4). On the other hand, no statistical difference was observed in risks in the increase of bone resorption markers among any group (Table 5). It is indicated that Natto intake more than three times per week may protect against a decline in markers of bone formation compared to no intake.
DISCUSSION

In this 1 y prospective study of premenopausal women, we observed a positive association between Natto intake and bone formation markers. On the other hand, Natto intake could not alter stiffness index regardless of its dose. Although bone formation markers such as BAP increased after 1 y and reduction of Glu was apparent at 6 mo, such alterations were not always consistent. Neither increment of Gla nor reduction in bone resorption marker, NTx/Cr, was significant. However, such monovariate analysis seemed to be insufficient to exclude confounding factors and to estimate which parameter was associated with bone turnover markers. Logistic regression analysis showed that Natto intake 3 times per week significantly prevented declining the level of markers of bone formation compared to no intake. Vitamin K2 is synthesized in intestinal flora so that its deficiency is less likely in adults and around 0.4ƒÊg/l of vitamin K2 is detected in subjects who do not take vitamin K2 rich food (15). Natto contains mainly around 9ƒÊg/g of MK-7, one of 14 isomers of vitamin K2 (24). Thus, Natto intake once per month and once per week reached serum levels of at most 0.9 and 2.8 µg/L, respectively (15). However, 3 times per week intake attained more than 100 µg/d of vitamin K2 in average so that its serum level reached as high as 8 µg/L (15), being 20 times as high as that of no intake. On the other hand, it has been suggested that 100–400 µg/d of vitamin K intake would assure nearly complete carboxylation of the bone Gla proteins (25). Natto intake 3 times per week alone accomplished as much vitamin K as this suggestion.

Since subjects in the present study took vegetables around recommended dietary allowance levels, their serum vitamin K1 concentration was supposed to be around 0.4–0.7 µg/L (15, 26). When the same amount of vitamin K1 from spinach and vitamin K2 from Natto are administered, peak serum concentration of vitamin K2 is about ten times higher than that of vitamin K1 (27). Moreover, vitamin K1 concentration reaches its peak at 6 h and is not detectable after 24 h, whereas vitamin K2 concentration in the circulation shows the peak-and-dome type pharmacokinetics (27). Vitamin K2 concentration reaches its peak at 8 h, and disappears slowly during the second part of the curve, while it remains detectable even after 72 h (27). Namely, because of a much shorter half-life as well as low absorption than those of vitamin K2, the effect of vitamin K1 through vegetables was negligible in the present study. In addition, the long half-life of vitamin K2 in the circulation ascertained to maintain serum vitamin K2 levels constant when Natto was taken at the frequency of 3 times per week, preventing the loss of bone formation.

Aside from vitamin K2, Natto contains high levels of calcium and isoflavones since it is made of soybean. Although isoflavones may exert an additional beneficial effect on bone health, this presumption appears to be less likely, based on the findings that Natto is the only soybean-derived food whose consumption correlated significantly with hip-fracture incidence (15). Calcium intake of the Japanese is less than 600 mg/d (28) and less than that of Caucasians (29). Since among dietary factors, daily calcium intake was considered to be the essential regulator of bone, it is possible that all Japanese may suffer from low calcium absorption. The subjects of the present study took only around 500 mg/d, even being lower than the Japanese average. However, soybean itself is commonly taken as well as soybean-derived foods, i.e., soy sauce, soybean paste (mis0, bean curd (tofu), fried bean curd, etc., so that Natto intake in the present study did not alter total calcium intake in each group, resulting in no significant findings on their Ca/Cr. On the other hand, since most Japanese are fond of fish, daily intake of vitamin D usually exceeds its recommended intake level in Japan (28) and the subjects in the present study also displayed high vitamin D intake. Such vitamin D intake may compensate the low calcium intake.

Longitudinal study proved the utility of menatrenone or MK-4 as a therapeutic agent for osteoporosis, because it suppresses the decrease in spinal BMD in postmenopausal women (30) and reduced the urinary excretion of calcium (31). Although epidemiological study showed that either vitamin K2 intake through Natto or vitamin K1 intake through lettuce had negative correlation with hip fracture incidence (15, 32), their dose-effect relationships remained unknown. Long observation and/or intervention will make its effect on
bone health more distinct. Moreover, aside from $\gamma$-carboxylation, vitamin K$_2$ reportedly induces apoptosis in leukemia cells, binds to nuclear factor of osteoblasts, etc. (33, 34). It also seems important to clarify the underlying mechanisms and/or whether such mechanisms are involved in the effect of vitamin K$_2$ on bone health. In any case, improvement in lifestyle including dietary intake may contribute to maintenance of bone stiffness in postmenopausal women.

Acknowledgments

We thank Mr. Tsutomu Higashimura, radiologist, and Miss Noriko Maeda, administrative dietitian, Kosei Hospital, Japan, for their technical support. This study was supported in part by a Grant-in-Aid for Scientific Research (C) (12670372) from the Ministry of Education, Science, Sports, Culture and Technology of Japan and also supported in part by a Research Project Grant (No. 11-404) from Kawasaki Medical School.

REFERENCES

1) Riggs BL, Melton III LJ. 1986. Involutional osteoporosis. N Engl J Med 314: 1676–1686.
2) U.S. Preventive Service Task Force. 2002. Screening for osteoporosis in postmenopausal women: Recommendations and rationale. Am Fam Physician 66: 1430–1432.
3) Brown JP, Josse RG. 2002. 2002 clinical practice guidelines for the diagnosis and treatment of osteoporosis in Canada. CMAJ 167: s1–s34.
4) Yamazaki K, Kushida K, Ohmura A, Sano M, Inoue T. 1994. Ultrasound bone densitometry of the os calcis in Japanese women. Osteoporosis Int 4: 220–225.
5) Luukinen H, Kakonen SM, Pettersson K, Koski K, Laipala P, Lovgren T, Kivela SL, Vaananen HK. 2000. Strong prediction of fractures among older adults by the ratio of carboxylated to total serum osteocalcin. J Bone Miner Res 15: 2473–2478.
6) Ozuru R, Sugimoto T, Yamaguchi T, Chihara K. 2002. Time-dependent effects of vitamin K$_3$ (menatetrenone) on bone metabolism in postmenopausal women. Endocr J 49: 363–370.
7) Iwamoto J, Takeda T, Ichimura S. 2000. Effect of combined administration of vitamin D$_3$ and vitamin K$_2$ on bone mineral density of the lumbar spine in postmenopausal women with osteoporosis. J Orthop Sci 5: 546–551.
8) Feskanich D, Weber P, Willett WC, Rockett H, Booth SL, Colditz GA. 1999. Vitamin K intake and hip fractures in women: a prospective study. Am J Clin Nutr 69: 74–79.
9) Sakano T, Notsumoto S, Nagaoaka T, Morimoto A, Fuji moto K, Masuda S, Suzuki Y, Hirauchi K. 1988. Measurement of K vitamins in food by high-performance liquid chromatography with fluorometric detection. Vitamins 62: 393–398 (in Japanese).
10) Tsukamoto Y, Ichise H, Kakuda H, Yamaguchi M. 2000. Intake of fermented soybean (Natto) increases circulating vitamin K$_2$ (menaquinone-7) and $\gamma$-carboxylated osteocalcin concentration in normal individuals. J Bone Miner Metab 18: 216–222.
11) Orimo H, Hosoda Y, Fujiwara S, Mizuno S, Hashimoto T, Tamaki T, Nose T, Yamamoto K, Sasaki R. 1991. Hip fracture incidence in Japan. J Bone Miner Metab 9: s89–s93.
12) Orimo H, Hashimoto T, Sakata K, Yoshimura N, Suzuki T, Hosi T. 2000. Trends in the incidence of hip fracture in Japan, 1987–1997: the third nationwide survey. J Bone Miner Metab 18: 126–131.
13) Ehara Y, Takahashi H, Hanahisa Y, Yamaguchi M. 1996. Effect of vitamin K$_2$ (menaquinone-7) on bone metabolism in the femoral-metaphyseal tissues of normal and skeletal-unloaded rats: enhancement with zinc. Res Exp Med 196: 171–178.
14) Koshihara Y. 1999. Vitamin K. Jpn J Clin Med 57: 2247–2253 (in Japanese).
15) Kanei M, Hedges SJ, Hosi T, Fujiwara S, Lyons A, Creun SJ, Ishida N, Nakagawa M, Takechi M, Sano Y, Mizuno Y, Hoshino S, Miyao M, Inoue S, Horiki K, Shiraki M, Ouchi Y, Orimo H. 2001. Japanese fermented soybean food as the major determinant of the large geographic difference in circulating levels of vitamin K$_2$: possible implications for hip-fracture risk. Nutrition 17: 315–321.
16) Takahashi K, Yoshimura Y, Katashima R. 1996. Development of food frequency and intake survey by questionnaire method. Bull Shikoku Univ 5: 23–35 (in Japanese).
17) Kaufman DW, Kelly JP, Wilhelm BE, Laszlo A, Sheehan JE, Koff RS, Shapiro S. 1999. The risk of acute major upper gastrointestinal bleeding among users of aspirin and ibuprofen at various levels of alcohol consumption. Am J Gastroenterol 94: 3189–3196.
18) Nelsestuen GL, Zytkovicz TH, Howard JB. 1974. The mode of action of vitamin K. Identification of $\gamma$-carboxyglutamic acid as a component of prothrombin. J Biol Chem 249: 6347–6350.
19) Shearer MJ. 1995. Vitamin K. Lancet 345: 229–234.
20) National Research Council. 1989. Recommended Dietary Allowances, 10th ed. National Academy Press, Washington, DC.
21) Morita R, Yamamoto I, Ito Y, Hamaoka Y, Ohta T, Takada M, Matsushita R, Masuda K. 1997. Quantitative ultrasound for the assessment of bone status. Osteoporos Int 7: S128–S134.
22) Takeda N, Miyake M, Kita S, Tomomitsu T, Fukunaga M. 1996. Sex and age pattern of quantitative ultrasound densitometry of the calcaneus in normal Japanese subjects. Calcif Tissue Int 59: 84–88.
23) Katsuyama H, Idemugi S, Fukunaga M, Sajioh K, Sunami S. 2002. Usual dietary intake of fermented soybeans (Natto) is associated with bone mineral density in postmenopausal women. J Nutr Sci Vitaminol 48: 207–215.
24) Yamaguchi M, Taguchi H, Gao YH, Igarashi A, Tsukamoto Y. 1999. Effect of vitamin K$_2$ (menaquinone-7) in fermented soybean (Natto) on bone loss in ovariectomized rats. J Bone Miner Metab 17: 23–29.
25) Rucker RB. 1997. Improved functional endpoints for use in vitamin K assessment: important implications for bone disease. Am J Clin Nutr 65: 883–884.
26) Kawana K, Takahashi M, Hoshino H, Kushida K. 2001. Circulating levels of vitamin K$_2$, menaquinone-4, and menaquinone-7 in healthy elderly Japanese women and patients with vertebral fractures and patients with hip fractures. Endocr Res 27: 337–343.
27) Schurgers LJ, Vermeer C. 2000. Determination of $\gamma$-carboxyglutamic acid and menaquinones in food: effect of food matrix on circulating vitamin K concentrations. Haemostasis 30: 298–307.
28) Health and Welfare Statistics Association. 2002. Daily Nutrient Intakes. *J Health Welfare Stat* **49**: 469 (in Japanese).

29) Dawson-Hughes B, Harris SS, Finneran S. 1995. Calcium absorption on high and low calcium intakes in relation to vitamin D receptor genotype. *J Clin Endocrinol Metab* **80**: 3657–3661.

30) Iwamoto I, Kosha S, Noguchi S, Murakami M, Fujino T, Douchi T, Nagata Y. 1999. A longitudinal study of the effect of vitamin K2 on bone mineral density in post-menopausal women: a comparative study with vitamin D3 and estrogen-progestin therapy. *Maturitas* **31**: 161–164.

31) Orimo H, Shiraki M, Fujita T, Onomura T, Inoue T, Kushida K. 1992. Clinical evaluation of menatetrenone in the treatment of involutional osteoporosis—a double blind multicenter comparative study with 1α hydroxy vitamin D3. *J Bone Miner Res* **7**: s122.

32) Feskanich D, Weber P, Willett WC, Rockett H, Booth SL, Colditz GA. 1999. Vitamin K intake and hip fractures in women: a prospective study. *Am J Clin Nutr* **69**: 74–79.

33) Yaguchi M, Miyazawa K, Otawa M, Katagiri T, Nishimaki J, Uchida Y, Iwase O, Gotoh A, Kawanishi Y, Toyama K. 1998. Vitamin K2 selectively induces apoptosis of blastic cells in myelodysplastic syndrome: flow cytometric detection of apoptotic cells using APO2.7 monoclonal antibody. *Leukemia* **12**: 1392–1397.

34) Hoshi K, Nomura K, Sano Y, Koshihara Y. 1999. Nuclear vitamin K2 binding protein in human osteoblasts; homologue to glyceraldehydes-3-phosphate dehydrogenase. *Biochem Pharmacol* **58**: 1631–1638.