Prolonged Intake of Dietary Fermented Soybeans (Natto) with the Reinforced Vitamin K₂ (Menaquinone-7) Enhances Circulating γ -Carboxylated Osteocalcin Concentration in Normal Individuals

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The change in circulating vitamin K₂ (menaquinone-7; MK-7) and γ -carboxylated osteocalcin (Gla osteocalcin) concentrations in normal individuals with the intake of fermented soybean (natto) was investigated. Forty eight volunteers (forty five males and three females) were divided into three groups of sixteen volunteers each (fifteen males and one female), and each group was given sequentially the fermented soybean (natto; 50 g) containing three different contents of MK-7 once a day for 14 d as follows: either regular natto with 865 μ g MK-7/100 g of natto, reinforced natto containing 1295 μ g MK-7/100 g, or 1730 μ g MK-7/100 g. Serum MK-7 was not found in normal individuals who had not had natto intake. Serum MK-7 and γ -carboxylated osteocalcin concentrations were significantly raised 7, 10, and 14 d after the start of the intake of reinforced natto containing 1295 or 1730 μ g MK-7/100 g. However, serum γ -carboxylated osteocalcin levels were not significantly elevated by the intake of regular natto, although serum-MK-7 levels were significantly raised. Moreover, serum γ -carboxylated osteocalcin concentration was significantly elevated 14 d after the intake of natto containing either 1295 or 1730 μ g MK-7/100 g, as compared with that of regular natto intake. The present study suggests that

the intake of dietary MK-7 in the reinforced natto can stimulate γ -carboxylation of osteocalcin, which plays an important role in bone formation in normal individuals.

Key words — vitamin K₂, menaquinone-7, γ -carboxylated osteocalcin, bone metabolism, osteoporosis

INTRODUCTION

Nutritional and pharmacological factors are important in preventing age-related bone loss. Previous clinical studies showed that vitamin K₂ may play a role in such prevention. Vitamin K₂ is essential for the γ -carboxylation of osteocalcin, a bone matrix protein containing γ -carboxyglutamic acids, which is synthesized in osteoblasts of bone tissues.^{1–3)} Vitamin K₂ is a fat soluble vitamin, and its intestinal absorption from the diet gradually declines with advancing age. Therefore, the serum levels of γ -carboxylated osteocalcin concentration in patients with osteoporosis may be decreased. The supplementation of vitamin K₂ may be very important as a preventive or therapeutic tool against osteoporosis.

Menaquinone-7 (MK-7) with seven isoprene units, one of analog of vitamin K₂, is abundant in fermented soybean (natto). It has been shown that serum MK-7 concentrations in women living in Tokyo, where the fermented soybean (natto) is consumed, is about ten times higher than the concentrations in those living in Europe.⁴⁾ An appropriate amount of MK-7 may be significant in preventing age-related bone loss, however, the biological effect of this substance in normal individuals has not yet been determined.

It was recently demonstrated that the prolonged dietary intake of MK-7 has a preventive effect on bone loss induced by ovariectomy in rats.^{5,6)} Moreover, it has been reported that the dietary intake of MK-7 for a short period may enhance γ -carboxylation of osteocalcin in the serum.⁷⁾ These observations support the view that dietary MK-7 may have a useful role in the prevention of osteoporosis on the basis of the direct promotion of γ -carboxylation of osteocalcin, which is important in the promotion of bone calcification.^{1,2)}

The present study was undertaken to determine the increase in serum MK-7 and γ -carboxylated osteocalcin concentrations in normal individuals following dietary intake for a longer period of fermented soybeans (natto) containing MK-7.

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Fig. 1. Study Protocol of Experiment

Dietary natto containing either 865, 1295 or 1730 μg MK-7/100 g was given to volunteers in Groups 1, 2 and 3 once a day for 14 d.

MATERIALS AND METHODS

Materials—— Regular natto was supplied by Asahi Freshia Co., Ltd. (Ibaragi, Japan), of which MK-7 content was measured to be 865 μg per 100 g of natto (wet weight). Reinforced natto with increased MK-7 content was made using fermenting soybean with a developed microorganism, a species of *Bacillus subtilis* OUV23481, which produces a high amount of MK-7 during the fermentation process. The contents of MK-7 in the reinforced natto were 1295 and 1765 μg per 100 g (wet weight).

Experimental Procedures—— Forty eight adults aged 24–46 years (forty five males and three females), who were judged to be healthy with no abnormal liver or kidney functions as assessed by standard biochemical data, were enrolled as volunteers in this study. Informed consent was obtained from all of them. The intake of other foods with an abundance of vitamin K was prohibited all volunteers during the period of the experiment. The time schedule of the experiment is shown in Fig. 1. The period for washout or intake of each type of natto was designed to be 14 d or 7, 10 and 14 d, respectively. The forty eight volunteers were divided into three groups sixteen volunteers each (fifteen males and one female). Each group was given sequentially once a day for 14 d fifty grams (wet weight) of fermented soybean (natto) containing three different amounts:

either 865, 1295 or 1730 μg of MK-7 per 100 g. The intake of natto was performed between 6 a.m. and 8 a.m. Blood samples were collected each at 10 a.m. and 12 a.m. on the day prior to the intake, or 7, 10 and 14 d after the intake. Serum MK-7, osteocalcin, calcium, inorganic phosphorus and alkaline phosphatase activity were measured using analytical methods (see the following).

Analytical Procedures—— Serum samples were obtained by centrifugation (2500 rpm for 5 min) between 20 and 40 min after blood collections, and then were stored at -20°C until assayed. Serum vitamin K_1 , MK-4 and MK-7 concentrations were measured by fluorescence detection high performance liquid chromatography.⁵⁾ Serum γ -carboxylated osteocalcin concentration was assayed using a Gla type Osteocalcin (Gla-OC) EIA Kit (Takara Shuzou, Shiga, Japan).⁸⁾ Serum calcium and inorganic phosphorus concentrations were determined using a Calcium E-HR KIT and an Inorganic Phosphorous-HR-II Kit, respectively (Wako Junyaku, Osaka, Japan).^{9,10)} Serum alkaline phosphatase activity was assayed using Clinimate ALP reagent (Daiichi Kagaku Yakuhin, Tokyo, Japan),¹¹⁾ and urinary pyridinoline and deoxypyridinoline concentrations were both measured using reversed-phase high-performance liquid chromatography.¹²⁾

Statistical Analysis—— Difference in values before and after the intake of each natto was estimated

Fig. 2. Changes in Serum MK-7 Concentrations Following Dietary Intake of Natto Containing MK-7

Dietary natto containing MK-7 in the three different amounts described in Fig. 1. Each value is expressed as the mean $\pm$ S.E.M. of nine to thirteen persons. # $p < 0.001$ as compared with the baseline values of natto intake in each group using Student's *t*-test. * $p < 0.05$ as compared with the values of natto intake of 865 μ g of MK-7/100 g at 7, 10 and 14 d using one-way ANOVA Tukey-Kramer and multiple comparison tests.

using Student's *t*-test. Paired *t*-test was used for the difference in values before and after the intake of each natto or between two groups at each intake period. In comparisons among multi-groups at each period of the intake, both tests were used, and one-way analysis of variance (ANOVA) Tukey-Kramer and multiple comparison tests were used in the difference among three groups. *P* values of less than 0.05 were considered statistically significant.

RESULTS

The change in serum MK-7 concentration in normal individuals following the start of intake of fermented soybeans (natto) containing the three different MK-7 contents is shown in Fig. 2. The intake of regular natto containing 865 μ g MK-7/100 g for 7, 10 and 14 d caused a significant elevation in serum MK-7 concentration; the values were increased from 0.85 ± 0.22 (before intake) to 34.25 ± 2.47 (7 d), 54.14 ± 3.37 (10 d) or 83.66 ± 4.63 (14 d) ng/ml, respectively. Serum MK-7 concentration was significantly enhanced by the intake of natto containing either 1295 or 1730 μ g MK-7/100 g for 7, 10 and 14 d. Serum MK-7 at 14 d after the start of intake was 110.31 ± 9.26 or 134.89 ± 9.17 ng/ml, respectively. Meanwhile, neither serum vitamin K₁ nor MK-4 was detected in any subject ingesting with

Fig. 3. Changes in Serum γ -Carboxylated Osteocalcin Concentrations Following Dietary Intake of Natto Containing MK-7

Dietary natto containing MK-7 in the three different amounts described in Fig. 1. Each value is expressed as the mean $\pm$ S.E.M. of nine to thirteen persons. # $p < 0.01$ and ** $p < 0.01$ as compared with the baseline values of natto intake in each group at 14 d using Student's *t*-test. * $p < 0.05$ as compared with the values of natto intake of 865 μ g of MK-7/100 g at 14 d using one-way ANOVA Tukey-Kramer and multiple comparison tests.

any of the three MK-7 amounts (data not shown).

Comparison among the three groups at each intake period containing either 865, 1295 or 1730 μ g MK-7/100 g showed significant differences among them at 7, 10 and 14 d after intake. The increase in serum MK-7 concentration was especially following natto intake containing either 1295 or 1730 μ g/100 g as compared with that of regular natto intake.

The change in serum γ -carboxylated osteocalcin concentration following natto intake is shown in Fig. 3. Regular natto did not cause a significant increase in serum γ -carboxylated osteocalcin concentration at 7, 10 and 14 d; the values were 7.830 ± 0.947 (before intake), 8.503 ± 0.772 (7 d), 8.810 ± 0.845 (10 d) or 9.318 ± 1.110 (14 d) ng/ml, respectively. Natto containing either 1295 or 1730 μ g MK-7/100 g caused a significant increase in serum γ -carboxylated osteocalcin concentration at 7, 10 and 14 d as compared with the values before intake: the values at 14 d were 12.627 ± 0.677 or 12.612 ± 0.786 ng/ml, respectively.

With respect to serum γ -carboxylated osteocalcin concentration, there was no significant difference among the three groups at 7 and 10 d after the intake of each natto. At 14 d, however, a significant difference was seen among the groups. In addition, a significant rise in serum γ -carboxylated osteocalcin concentration was observed following the intake of natto containing either 1295 or 1730 μ g MK-7/100 g,

as compared with that of regular natto.

Serum calcium and inorganic phosphorus concentrations were significantly elevated 14 d after the intake of natto containing 1730 μg MK-7/100 g, as compared with the value prior to ingestion (data not shown). Serum alkaline phosphatase activity and urinary pyridinoline and deoxypyridinoline concentrations, a marker of bone resorption, were not significantly altered by the natto intake (data not shown).

DISCUSSION

Vitamin K is essential for γ -carboxylated osteocalcin concentration, which is synthesized in osteoblasts.^{1,2)} Undercarboxylated osteocalcin has very poor affinity to hydroxyapatite as compared with γ -carboxylated osteocalcin in bone tissue.³⁾ There are two types of vitamin K: vitamin K₁ and K₂. Though vitamin K₁ is a single compound, vitamin K₂ is a series of vitamins with multiisoprene units at the 3-position of naphthoquinone. MK-4, with four isoprene units, has been approved as a therapeutic agent for osteoporosis in Japan.^{13,14)} MK-7, on the other hand, is a natural vitamin K₂ and is abundant only in fermented soybean (natto). MK-7 has been shown to have an anabolic effect on bone calcification as true of MK-4.^{15,16)}

It has been demonstrated that ovariectomy-induced bone loss in rats is prevented by the prolonged feeding of natto at a low level of 18.8 μg of MK-7/100 g diet for 150 d.^{5,6)} More recently, it has been reported that serum γ -carboxylated osteocalcin concentration is raised in normal individuals by the dietary intake of reinforced natto containing MK-7 of more than 650 $\mu\text{g}/\text{d}$.⁷⁾ Thus, the intake of dietary MK-7 may be a very useful tool in the prevention of osteoporosis with aging. Furthermore, the present study supports the view that longer intake of fermented soybean (natto) containing MK-7 may stimulate bone calcification in normal individuals.

We used a regular natto containing 865 μg MK-7/100 g and a reinforced natto of either 1295 or 1730 μg MK-7/100 g, which was made by the fermentation of soybean using the developed microorganism, *Bacillus subtilis* OUV23481. Forty eight volunteers (forty five males and three females) were divided into three groups of sixteen volunteers each. Each group was sequentially given fifty grams of one of the reinforced natto foods described above once a day for 14 d. The dietary intake of natto con-

taining either 1295 or 1730 μg of MK-7/100 g for 7, 10 and 14 d caused a significant elevation of serum γ -carboxylated osteocalcin concentration, although such an effect was not seen by the intake of regular natto. This finding suggests that the dietary intake of an appropriate amount of MK-7 of more than 430 μg per day is needed to stimulate the γ -carboxylated osteocalcin in bone matrix. Perhaps a certain level of serum MK-7 may be essential for a significant rise in serum γ -carboxylated osteocalcin concentration. Vitamin K₁ and MK-4 were not seen in the serum during the intake of reinforced natto. MK-7 itself may directly stimulate the γ -carboxylated osteocalcin in osteoblasts of bone tissues.

Prolonged intake of MK-7 of less than an appropriate amount may not cause any significant elevation of serum γ -carboxylated osteocalcin concentration. The serum levels may be significantly increased by the sequential intake of an appropriate amount of MK-7 of more than 430 μg per day for longer than 7 d. Perhaps γ -carboxylated osteocalcin formed in the osteoblasts of bone tissues can be metabolized by turnover in the lining and consequently may reach a certain stationary level. Serum MK-7 levels seen by the intake of regular natto may not reach critical levels to induce a significant increase in γ -carboxylated osteocalcin concentration. An appropriate amount of dietary MK-7 may need to be supplied sequentially to maintain a level sufficient to significantly enhance of serum γ -carboxylated osteocalcin concentration. Reinforced natto intaken at more than 1295 μg MK-7/100 g may be useful to increase serum γ -carboxylated osteocalcin concentration, which stimulates bone mineralization in normal individuals.

Epidemiological data reportedly indicates that the intake of natto may play a role in the prevention of osteoporosis.⁴⁾ However, it has not been determined whether the prolonged intake of dietary natto causes a rise in MK-7 and a corresponding increase in γ -carboxylated osteocalcin in the serum of normal individuals. The appropriate amount of dietary MK-7 may have a stimulatory effect on bone mineralization in these individuals. The intake of reinforced natto which contains more MK-7 than does regular natto may participate in the prevention of age-related bone loss.

REFERENCES

- 1) Hauschka P.V., Lian J.B., Gallop P.M., *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3925–3929 (1975).
- 2) Hauschka P.V., Carr S.A., *Biochem. J.*, **21**, 2538–2547 (1982).
- 3) Price P.A., *Vitam. Horm.*, **42**, 65–108 (1985).
- 4) Hosoi T., *Nippon Ronnen Igakkai Zasshi*, **33**, 240–244 (1996).
- 5) Yamaguchi M., Taguchi H., Gao Y.H., Tsukamoto Y., *J. Bone Miner. Metab.*, **17**, 23–29 (1998).
- 6) Yamaguchi M., Kakuda H., Gao Y.H., Tsukamoto Y., *J. Bone Miner. Metab.*, **18**, 71–76 (2000).
- 7) Tsukamoto Y., Ichise H., Yamaguchi M., *J. Bone Miner. Metab.*, in press (2000).
- 8) Koyama N., Ohara K., Yokota H., Kurone T., Katayama M., Hino F., Kato I., Akai T., *J. Immunol. Methods*, **17**, 17–23 (1991).
- 9) Kitano M., Ueda J., *Nippon Kagaku Zasshi*, **92**, 168–170 (1971).
- 10) Daly J.A., Ertingshausen G., *Clin. Chem.*, **18**, 263–265 (1972).
- 11) Hausamen T.U., Helger R., Rick W., Gross W., *Clin. Chim. Acta*, **15**, 241–245 (1967).
- 12) Pratt D.A., Daniloff Y., Duncan A., Robins S.P., *Anal. Biochem.*, **207**, 168–175 (1992).
- 13) Boukaert H.J., Said A.H., *Nature* (London), **19**, 849 (1960).
- 14) Price P.A., Williamson M.K., Lothringer M.K., Lothringer J.W., *J. Biol. Chem.*, **256**, 12760–12766 (1981).
- 15) Sato T., Isobe Y., Ehara Y., Yamaguchi M., *Vitamins*, **70**, 317–322 (1996).
- 16) Ehara Y., Takahashi H., Hanahisa Y., Yamaguchi M., *Res. Exp. Med.*, **196**, 171–178 (1996).