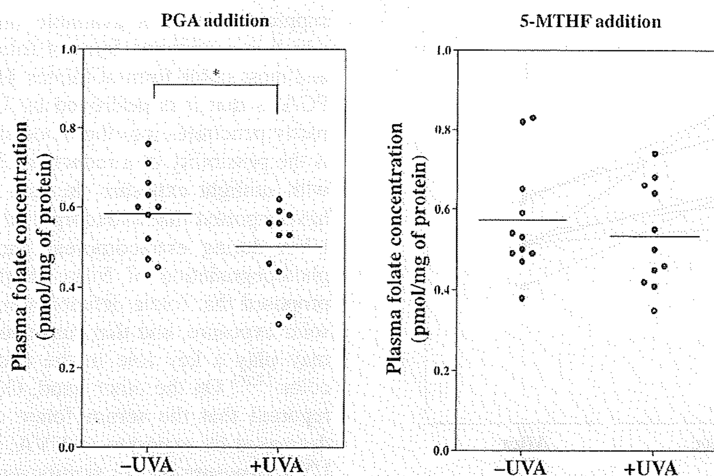

Fig. 5. Change of the Folate Concentration in Human Blood, to Which PGA or 5-MTHF Had Been Added, by UVA Irradiation (Experiment 4, *in vitro* experiment).

Blood was taken from the venous vein. One milliliter of 110 μM PGA or 110 μM 5-MTHF was added to 6 ml of the blood taken and mixed well. Three milliliter of the sample was withdrawn from the PGA- or 5-MTHF-added blood and irradiated with UVA light for 120 min. The UVA dose was 3,200 mJ/cm^2. As a control, the blood in a dish was placed in the dark. The folate was measured by a microbioassay. Circles indicate individual values, and the horizontal line in the figure is the mean. *Statistically significant at $p < 0.05$.

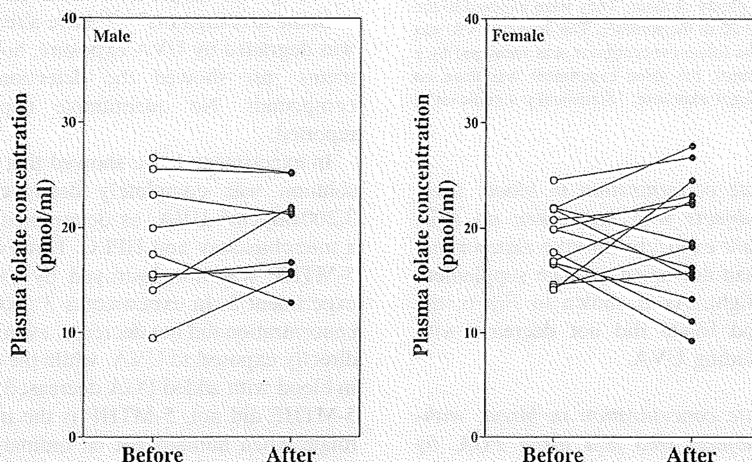

Fig. 6. Change of the Folate Concentration in Human Blood, Which Was Withdrawn from the Subjects Who Had Not Taken a PGA Supplement, by UVA Irradiation (Experiment 6, *in vivo* experiment).

The subjects were 9 male and 14 female students who ate freely. The blood was taken from the venous vein before and after sunlight exposure at 11:00 and 13:00, respectively. The folate concentration was measured by a microbioassay.

tion did not change with UVA irradiation as shown in Fig. 4.

Change of the folate concentration in human blood which PGA or 5-MTHF added to the blood with UVA irradiation in vitro *(Experiment 4)*

PGA or 5-MTHF was directly added to human blood, and the blood was exposed to UVA. As is shown in Fig. 5, the folate concentration in the PGA-added blood was significantly decreased with UVA irradiation, while that in the 5-MTHF-added blood did not decrease.

Comparison of the human blood folate concentrations between males and females who ate freely (Experiment 5)

The concentrations of blood folate in humans who ate freely, but had not taken folate as a supplement, were measured. We have previously reported that the concentration was higher in females than in males.[8] In the present experiment, the difference between males and females was not significant as shown in Table 1.

Table 1. Comparison of the Serum Folate Concentration between Male and Female Japanese Young Adults Who Ate Freely (Experiment 5)

	Mean	SD	Minimum	Maximum	Medium
Male (n = 23)					
Folates in serum (pmol/ml)	15.0	5.8	7.2	29.2	13.8
Female (n = 32)					
Folates in serum (pmol/ml)	17.7	5.9	9.5	31.5	15.7

The subjects were 23 male and 32 female students, who ate freely. Blood was taken from the venous vain before lunch at around 12:00. The folate concentration was measured by a microbioassay.

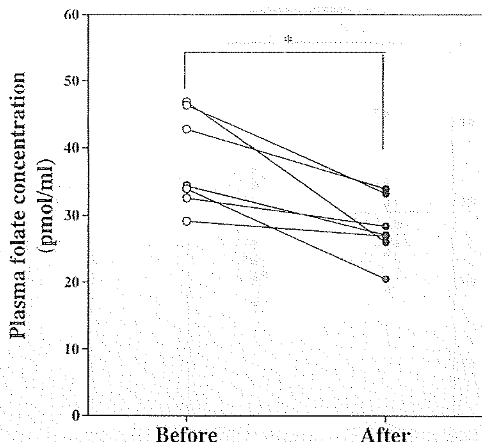

Fig. 7. Change of the Folate Concentration in Human Blood, Which Was Withdrawn from the Subjects Who Had Taken PGA, by Sunlight Exposure (Experiment 7, *in vivo* experiment).

The subjects were 7 female students who ate freely. The PGA preparation (0.25 mg) was administered to them at each meal for two days before and at breakfast on the blood collection day. The blood (3 ml) was taken from the venous vein before and after sunlight exposure at 11:00 and 13:00, respectively. The subjects wore shorts and a tank top to expose plenty of skins. They were exposed to the sunlight from 11:00 to 13:00 in the summer. The dose of UVA was about 12,000 mJ/cm^2. The folate concentration was measured by a microbioassay. As a control, the same experiment was done on another day without sunlight exposure. *Statistically significant at $p < 0.05$.

*Change of the folate concentration in blood, withdrawn from those subjects who had taken no PGA supplements, by sunlight exposure (*in vivo *experiment)*

The subjects who had not taken a folate supplement were exposed to sunlight. As is shown in Fig. 6, the concentration of blood folate did not decrease with sunlight exposure including UVA.

*Change of the folate concentration in blood, withdrawn from those subjects who had taken PGA, by sunlight exposure (*in vivo *experiment)*

The subjects who had been administered with PGA were exposed to sunlight. As is shown in Fig. 7, the concentration of blood folate significantly decreased with sunlight exposure (38.0 ± 7.2 pmol/ml *vs.* 28.1 ± 4.6 pmol/ml). On the contrary, the blood folate concentration in the subjects not exposed to sunlight was not altered (38.6 ± 3.3 pmol/ml *vs.* 38.2 ± 2.3 pmol/ml in 11:00 *vs.* 13:00). In other words, the blood folate concentration was decreased by 74% with sunlight exposure.

Discussion

Folate, a B-group vitamin, is the essential cofactor in the biosynthesis of a *de novo* purine base. So, the rate of folate catabolism progressively increases during pregnancy and the folate demand increases. For a example, Higgins *et al.*[14] have reported that the requirements of folate in the second trimester and in the third trimester were 430 and 540 µg/d, while that in nonpregnant women was 250 µg/d. Some investigators[15,16] have also advised women to consume a folate supplement for the duration of pregnancy and lactation. The main folate supplement that is available on the market is PGA, which is a synthetic form of folate, and is the oxidized and most stable form of folates. However, the demerit of PGA is that it is destroyed by UVA exposure[6] which partly penetrates into the blood stream. Therefore, there is the possibility of a reduction of the blood folate level with sunlight exposure. In fact, Der-Petrossian *et al.*[7] have reported that extracorporeal exposure of plasma to UVA during extracorporeal photophoresis led to the photodegradation of folate. Furthermore, it has been proposed that folate deficiency may result from intense solar exposure, and that sun-induced folate degradation may play a key role in the evolution of human skin color.[17,18] On the other hand, Gambichler *et al.*[9] have reported that the serum folate concentration was not decreased by exposure to UVA; their data suggest that UVA exposure do not significantly influence the serum folate level of healthy subjects and they concluded that the neural tube defects claimed to occur after per conceptual UVA exposure were probably not due to UVA-induced folate deficiency. Therefore, no definite conclusion about the biological significance of folate photodegradation *in vivo* can yet be drawn. We carried out the present experiment to elucidate this controversy.

Some investigators[6,7,19] have already shown that PGA was degraded by UVA exposure, however, these experiments just showed the detection of the degraded compounds. No quantitative experiment had been reported.

In experiment 1, we showed that PGA in an aqueous solution was completely destroyed by exposure to 3,200 mJ/cm^2 UVA, as determined by the methods of a microbioassay and HPLC. However, we showed that 5-MTHF was not destroyed by exposure to UVA in experiment 2. In experiments 3 and 4, the blood folate concentration did not decrease, even when the blood was directly exposed to UVA, while the folate concentration in blood with added PGA decreased and that with added 5-MTHF did not. 5-MTHF is the major form of blood folate when humans eat an ordinary food. From these findings, it is suggested that the blood folate level decreased when the blood contained PGA, while the blood folate level did not decrease when the blood did not contain PGA. We have already reported that the female blood folate level was higher than that of males when the subjects were fed on a semi-purified diet containing PGA.[8] However, as shown in Table 1 (experiment 5), the blood folate levels between males and females were no different when the subjects ate freely and had not taken PGA. From these data, we hypothesize that PGA in the blood is destroyed, but not 5-MTHF, by exposure of sunlight. If humans do not take PGA, the blood folate concentration would not be decreased by exposure to sunlight, because the form of blood folate is mainly 5-MTHF. On the contrary, if humans take PGA, the blood concentration would be decreased by exposure to sunlight, because the PGA taken appears in the blood stream as PGA itself. Thus, we carried out experiments 6 and 7. As was anticipated, when the subjects who had taken no PGA supplement were exposed to sunlight, the blood folate level did not decrease, while when the subjects who had taken PGA were exposed to sunlight, the folate level was significantly decreased (Fig. 8).

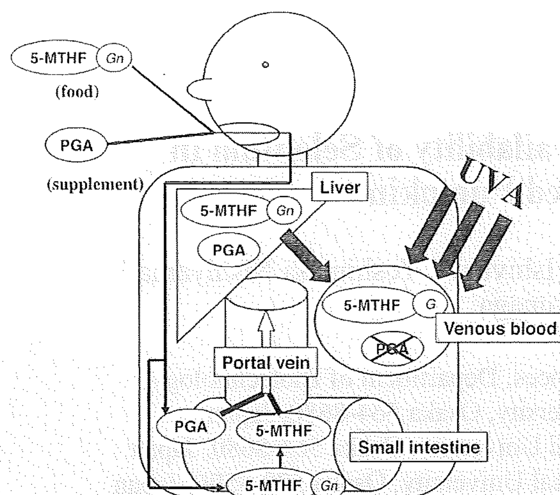

Fig. 8. Different Fates of PGA and 5-MTHF in the Blood Stream When Humans Are Exposed to Sunlight.

When humans take biological food concomitant with PGA as a folate supplement, 5-MTHG and PGA appear in the blood stream. Under such conditions, if humans are exposed to a sufficient amount of sunlight, only PGA is destroyed, so the blood folate level decreases with sunlight exposure. When humans take only biological food, only 5-MTHF-G is present in the blood, so even when humans are exposed to a sufficient amount of sunlight, the blood folate level does not decrease. 5-MTHF-Gn, 5-methyltetrahydrofolate polyglutamic acid.

In conclusion, only PGA, a synthetic form of folate in the blood stream was destroyed by UVA, but not 5-MTHF. We recommend that 5-MTHF is superior to PGA as a folate supplement.

Acknowledgment

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Short Communication

Assessment of Nutritional Availability of Selenium in Selenium-enriched Pumpkin

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Abstract

The nutritional availability of selenium (Se) in Se-enriched pumpkin (Se-pumpkin) was assessed by comparing with selenite and Se-enriched *Kaiware* radish sprouts (Se-sprouts). Male weanling ddY mice were fed a *Torula* yeast-based Se-deficient diet. After feeding for 3 weeks, mice were divided into 7 groups and fed the basal diet or a diet supplemented with 0.05 or 0.25 μg/g of Se as either sodium selenite, Se-pumpkin or Se-sprouts for further one week. Supplementation of Se dose-dependently increased serum and liver Se concentrations and glutathione peroxidase (GPX) activities. In serum Se and GPX, the increases by Se supplementation did not significantly vary with Se source, but in the liver Se and GPX, the increases by selenite supplementation were significantly higher than those by supplementation with Se-pumpkin or Se-sprouts. A difference between the effect of Se-pumpkin and that of Se-sprouts was found in the elevation of liver Se concentration ; supplementation with Se-pumpkin caused significantly higher elevation of liver Se than that with Se-sprouts. When liver Se was used, the nutritional availabilities of Se from Se-pumpkin and that Se-sprouts were estimated to be 97% and 65% to selenite Se, respectively. However, when liver GPX was used for the estimation, the availability of Se from either Se-pumpkin or Se-sprouts was less than 50% to selenite Se.

Keywords : selenium, nutritional availability, glutathione peroxidase, selenium-enriched vegetables, selenium-enriched radish sprouts, selenium-enriched pumpkin

Introduction

Selenium (Se) is an essential trace element in human and animal nutrition, and plays several important roles in the form of selenoproteins that include the families of glutathione peroxidase (GPX), deiodinases or thioredoxine reductases [1]. Besides the nutritional roles, Se is thought to be associated with cancer prevention from the results of many epidemiological studies and animal experiments [2]. To prevent a low Se status, various Se-enriched foods were prepared and used to increase daily Se intake [3, 4]. In particular, several Se-enriched plant foods have been developed since their anti-tumor activities are expected to be higher than those of selenite, selenate or high Se yeast [4].

The utilization of dietary minerals including Se is the net result of several physiological and metabolic processes that converted a portion of ingested minerals to certain metabolically critical forms that are necessary for normal physiological function. In the view of mineral nutrition, it is necessary to show an extent of the biological utilization of dietary minerals for their critical or functional forms quantitatively. The quantitative description of biological utilization of dietary minerals has come to

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be called their "bioavailability" or "nutritional availability" [5]. There are three factors contributing to the nutritional availability ; physiological characteristics of host animals, dietary components ingested with minerals and chemical species of minerals. Since the composition of Se species in the Se-enriched plant foods is diverse [6], their nutritional availabilities are thought to vary with the kinds of plant species. In the present study, we attempted to compare the nutritional availability of Se in Se-enriched pumpkin (Se-pumpkin) to those in selenite and Se-enriched *Kaiware* radish sprouts (Se-sprouts) by using tissue Se deposition and GPX activity of mice given these Se sources.

Materials and Methods

Se-pumpkin was purchased from PhytoSelenium Research Laboratories (Kumamoto, Japan) [7], and Se-sprouts were prepared by hydroponics, which used 10 μg Se/ml of sodium selenite solution as described previously [8]. Both Se-enriched vegetables were freeze-dried and milled. Se contents of Se-pumpkin and Se-sprouts were 45 and 121 μg/g dry weight, respectively.

The protocol of the animal experiment was reviewed and approved by the Animal Ethics Committee of Kansai Medical University. Fifty-six male weanling ddY mice were fed a *Torula* yeast-based Se-deficient basal diet [9]. After feeding for 3 weeks, mice were divided into 7 groups and fed the basal diet or the basal diet supplemented with 0.05 or 0.25 μg/g of Se as either sodium selenite, Se-pumpkin or Se-sprouts for a further week. Serum and liver were then collected, and their Se contents and GPX activities were measured. Se was determined by inductively coupled plasma mass spectrometry [10] and GPX activities were assayed using *tert*-butyl hydroperoxide as the peroxide substrate [11].

In the present study, the nutritional availability of Se from Se-pumpkin or Se-sprouts was assessed using sodium selenite as reference Se. The concentration of Se and GPX activity in liver and serum were used as the responses to increasing amounts of dietary Se. As the responses (Y) to increasing amounts of dietary Se (X) can be described by the general equation $Y = m X + k$, the relative nutritional availability of Se from Se-enriched vegetables was estimated by a slope-ratio technique that compares the slope of dose-response plots to the slope observed for selenite Se [12].

Results

No significant difference was observed in the body weight among groups. At the end of the feeding period, the mean ± SD of body weight for all mice (n=56) was 33.2 ± 1.9 g.

Se concentration and GPX activities in the liver and serum are summarized in Tables 1. The Se concentration and GPX activities both increased gradually with an increase of the supplementary level of Se, regardless of its source or the tissue monitored. In the serum Se and GPX, the increases by Se supplementation did not vary with the Se source, but in the liver Se and GPX, the increases by selenite supplementation were higher than those supplemented with Se-pumpkin or Se-sprouts. In the elevation of liver Se concentration, supplementation with Se-

Table 1 Se concentration and GPX activities in serum and liver of rats fed experimental diets

Se supplemented to diet		Se concentration		GPX activity	
Source	Level (μg/g)	Serum (ng/ml)	Liver (ng/g)	Serum (unit/ml)	Liver (unit/g protein)
None	-	37 ± 5 ^{a}	54 ± 3 ^{a}	0.17 ± 0.01 ^{a}	17 ± 3 ^{a}
Selenite	0.05	162 ± 10 ^{b}	210 ± 20 ^{b}	0.36 ± 0.07 ^{ab}	75 ± 11 ^{a}
Se-pumpkin	0.05	113 ± 6 ^{b}	98 ± 3 ^{ab}	0.22 ± 0.03 ^{a}	24 ± 6 ^{a}
Se-sprouts	0.05	102 ± 4 ^{ab}	92 ± 3 ^{ab}	0.20 ± 0.03 ^{a}	16 ± 5 ^{a}
Selenite	0.25	449 ± 20 ^{c}	657 ± 43 ^{d}	0.81 ± 0.12 ^{c}	442 ± 35 ^{c}
Se-pumpkin	0.25	428 ± 24 ^{c}	598 ± 36 ^{d}	0.81 ± 0.13 ^{c}	212 ± 42 ^{b}
Se-sprouts	0.25	411 ± 23 ^{c}	463 ± 48 ^{c}	0.72 ± 0.13 ^{bc}	217 ± 42 ^{b}

Values are the means ± SEM (n=8). GPX units expressed as μmol NADPH oxidized per min. Means in the same column not sharing a common superscript differ significantly ($p < 0.05$) by analysis of variance followed by Tukey-Kramer multiple range test.

Table 2 Regression of supplementary Se level (X) with parameters of Se status (Y) and nutritional availability of Se from Se-pumpkin or Se-sprouts.

Parameters	Source of Se supplemented	Regression	Correlation coefficient (r)	Nutritional availability (%)
Serum Se	Selenite	Y = 1592 X + 56	0.973	-
	Se-pumpkin	Y = 1568 X + 36	0.975	98.5
	Se-sprouts	Y = 1362 X + 36	0.911	85.6
Serum GPX	Selenite	Y = 2.46 X + 0.20	0.767	-
	Se-pumpkin	Y = 2.67 X + 0.14	0.815	108.5
	Se-sprouts	Y = 2.06 X + 0.14	0.711	83.7
Liver Se	Selenite	Y = 2356 X + 71	0.954	-
	Se-pumpkin	Y = 2267 X + 23	0.967	96.2
	Se-sprouts	Y = 1529 X + 38	0.858	64.9
Liver GPX	Selenite	Y = 1735 X + 5	0.952	-
	Se-pumpkin	Y = 819 X + 3	0.804	47.2
	Se-sprouts	Y = 757 X + 2	0.746	43.6

Regression was fitted to the equation $Y = m X + k$, where Y represented the parameters in mice fed the basal diet or the diet supplemented with Se at X level (μg/g). Units of parameters are the same as in Table 1. Nutritional availability was estimated using the slope of the regression ; (slope of Se-pumpkin or Se-sprouts)/(slope of selenite) x 100.

pumpkin caused a higher elevation of liver Se than that with Se-sprouts.

The regression analyses of supplementary Se with the Se concentration or GPX activities are summarized in Table 2. As the increases of Se concentration and GPX activity in mouse serum or liver were significantly correlated with supplementary levels of each Se source, linear regression could be calculated in each combination. Accordingly, as also described in Table 2, the relative nutritional availability of Se from Se-pumpkin or Se-sprouts can be estimated by the slope ratio analysis, which uses sodium selenite as a reference. Based on the serum parameters, the availability of Se from either Se-pumpkin or Se-sprouts was more than 80% to selenite Se. However, based on the liver GPX, the availability of Se from either Se-pumpkin or Se-sprouts was less than 50% to selenite. When liver Se was used for analysis, the nutritional availability of Se from Se-pumpkin and that from Se-sprouts was estimated to be 97% and 65% to selenite Se, respectively.

Discussion

In the estimation of nutritional availability, each parameter of Se status gave difference values. Among the responses of parameters in mice supplemented with selenite, serum GPX gave the lowest correlation coefficient. Compared to liver GPX activity, serum GPX activity reaches a plateau level at lower dietary Se level [13]. Thus, this low correlation indicates a possibility that the response of serum GPX reached a plateau level in the tested range of Se supplemented. When comparison between liver GPX and Se concentration is made, GPX has been thought to be superior to Se concentration as an index for Se status because GPX is one of the functional forms of Se in tissues [5]. Accordingly, nutritional availability based on the liver GPX is the most reliable among the four parameters, and the availability of Se either from Se-pumpkin or Se-sprouts is less than 50% to selenite Se.

We have already identified the main Se species in Se-pumpkin and Se-sprouts as protein-bound selenomethionine (SeMet) [14] and *Se*-methylselenocysteine (MeSec) [8], respectively. Dietary Se must be metabolized to selenide before incorporation to selenoproteins [1]. The low nutritional availability of Se from Se-pumpkin or Se-sprouts estimated in the present study indicates that the formation of selenide either by demethylation of MeSec or transsulfuration of SeMet was not sufficient for the synthesis of selenoprotein including GPX.

When liver Se was used in the estimation of nutritional availability, Se from Se-pumpkin gave higher availability values than that from Se-sprouts. Since SeMet is a non-specific form of Se that is metabolized as a constituent of methionine pool, SeMet can be non-specifically incorporated into body proteins [15, 16]. Accordingly, this result

may indicate that SeMet in Se-pumpkin was nonspecifically incorporated into liver protein. The SeMet incorporated to liver protein can take part in the amino acid pool, be metabolized to selenide *via* the transsulfuration pathway gradually and then be incorporated into selenoproteins. Thus, with the long-term administration of Se-pumpkin, the nutritional availability of Se from Se-pumpkin may be higher than that obtained in the present study.

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原　著

誘導体化とガスクロマトグラフィー －質量分析によるセレン強化食品中の含セレンアミノ酸の同定－

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Identification of Selenoamino Acids in Selenium-enriched Foods by Derivatization and Gas Chromatography-Mass Spectrometry

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Summary

Selenoamino acids in selenium (Se)-enriched foods were identified by gas chromatography-mass spectrometry (GC-MS) after derivatization using a commercial amino acid analysis kit (EZ : faast™). After the derivatization, a compound eluted at the same retention time as derivatized *Se*-methylselenocysteine (MeSec) in GC was detected in Se-enriched *Kaiware* radish sprouts and Se-enriched garlic bulb. Mass spectrum of this derivatized compound was coincident with that of derivatized MeSec ; MeSec was identified in these Se-enriched foods. Similarly, selenomethionine was identified in Se-enriched yeast using EZ : faast™ and GC-MS. Analysis by high performance liquid chromatography-inductively coupled plasma mass spectrometry showed a presence of selenohomolanthionine (SeHL) in Se-enriched mung bean sprouts. However, SeHL could not be analyzed by GC-MS after the derivatization by EZ : faast™.

疫学調査や動物実験によって，必須微量元素のセレンには抗腫瘍作用のあることが明らかにされている[1]。しかし，セレン化合物は毒性も強いため，ヒトへの応用は進んでいない。セレンはイオウの同族元素であり，自然界には含硫アミノ酸のセレンアナログである含セレンアミノ酸が存在している。とくに，セレンを蓄積した植物には，*Se*-メチルセレノシステイン（MeSec）をはじめとする多様な含セレンアミノ酸が存在する[2,3]。われわれは，MeSecを豊富に含有するセレン強化カイワレダイコンスプラウトが亜セレン酸に比較してセレンとしての栄養有効性は低いが，抗腫瘍活性は高いことを示した[4,5]。このようにセレン強化植物には，機能性の高い含セレンアミノ酸が存在しており，高セレン環境下で野菜類を栽培する試みが世界各地で行われている[6]。

セレン化合物の同定には，誘導結合プラズマ質量分析（ICPMS）を検出に用いた高速液体クロマトグラフィー（HPLC）や液体クロマトグラフィー－質量分析（LC-MS）が頻用されている。前者ではセレン化合物を特異的に検出でき，後者では未知化合物の構造を推定できる。しかし，HPLC-ICPMSが有機溶媒，LC-MSが不揮発性の溶媒をそれぞれ嫌うことから，両者を共通のカラム－溶媒系で実施することは困難である。したがって，セレン化合物の同定においてHPLC-ICPMSとLC-MSは独立して用いられており，食品や生体中のセレン化合物の同定を効率よく進めることは現在でも難しい。

一方，ガスクロマトグラフィー－質量分析（GC-MS）は，未知化合物の同定技術として古くから確立している。含セレンアミノ酸のような非揮発性化合物の場合，GC-MSで分析するには誘導体化処理を行うことが必要である。近年，生体や食品中の遊離アミノ酸を固相抽出後，誘導体化処理するキット（アミノ酸誘導体化キット）が開発されており，アミノ酸類のGC-MS分析を簡便かつ短時間で実施することが可能となっている。今回，セレン強化食品中の含セレンアミノ酸をこのようなアミノ酸誘導体化キットとGC-MSを用いて同定することに成功したので報告する。

実験方法

1. 試料など

セレン強化カイワレダイコンスプラウトとセレン強化リョクトウスプラウトは，既報[2]に記載した方法に従って栽培したものを用いた。セレン強化ニンニク鱗茎は植物セレニウム研究所（熊本，阿蘇町）から購入した。セレン強化酵母乾燥粉末はBiospringer社（フランス）が生産した

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ものを光洋商会（東京）から購入した。セレン強化酵母以外の試料は，凍結乾燥後，ミルで粉末とした。各試料のセレン含量（μg/g dry weight）は以下のとおりである。セレン強化カイワレダイコン，82.3；セレン強化リョクトウスプラウト，16.7；セレン強化ニンニク，226；セレン強化酵母，1154。

実験に使用した試薬中，セレノホモランチオニン（SeHL）は千葉大学薬学部衛生化学教室から供与されたものを使用した。その他の試薬は市販のものを用いた。また，アミノ酸誘導体化を行うためのアミノ酸分析キットEZ：faast™（Phenomenex社，米国）は島津GLC（京都）より購入した。

2. 含セレンアミノ酸の抽出

セレン強化酵母以外の試料については，乾燥粉末100 mgに50％エタノール5 mLを加え，十分に撹拌した後，遠心分離して抽出液を調製し，分析用の試料とした。抽出液へのセレンの抽出率（％）は以下のとおりであった。セレン強化ニンニク，85.2；セレン強化カイワレダイコンスプラウト，80.9；セレン強化リョクトウスプラウト，61.0。

セレン強化酵母は既報[7]に従って，乾燥酵母100 mgを5 mLの蒸留水中で10 mgのプロテアーゼXIV®（Sigma-Aldrich社，米国）を用いて室温（約25℃）で24時間加水分解処理を行い，遠心分離して得られた抽出液を分析用の試料とした。抽出液へのセレンの抽出率は85.4％だった。

3. キットによる含セレンアミノ酸の誘導体化とGC-MSによる分析

各抽出液100 μLにアミノ酸分析キットであるEZ：faast™を用いて誘導体化処理を行い，GC-MS用の試料を調製した。GC-MSの分析条件は以下のとおりである。機器，Parvum 2（島津，京都）；カラム，Zebron ZB-AAA（Phenomenex社，米国）；キャリアガス，ヘリウム；流量，1.1 mL/min；気化温度，250℃；カラム温度，110～320℃（30℃/min昇温）；分析時間，7分；試料注入量，2 μL；イオン源温度，240℃；スキャン範囲，45～450 m/z；サンプリング速度，3.5 scan/s。

4. HPLC−ICPMSによる分析

リョクトウスプラウト抽出液をHPLC-ICPMSで分析し，含有されるセレン化合物の分子種を推定した。分析条件は以下のとおりである。カラム，Develosil RP-Aqueous（野村化学）；移動相，0.1％トリフルオロ酢酸；試料注入量，20 μL；流速，0.5 mL/min；検出器，島津ICPM-8500；検出質量数，77, 78, 82。

結果と考察

1. セレン強化カイワレダイコンとセレン強化ニンニク

すでに，HPLC-ICPMSを用いた分析においては，セレン強化カイワレダイコンとセレン強化ニンニク中のセレンの主要な分子種がMeSecであることが示されている[2,3]。そこで本実験では，セレン強化カイワレダイコンとセレン強化ニンニク中に存在すると考えられるMeSecを誘導体化後，GC-MSを用いて同定することを試みた。

Fig. 1は，EZ：faast™を用いて誘導体化したMeSec，誘導体化処理したセレン強化カイワレダイコンスプラウト抽出液，および誘導体化処理したセレン強化ニンニク抽出液

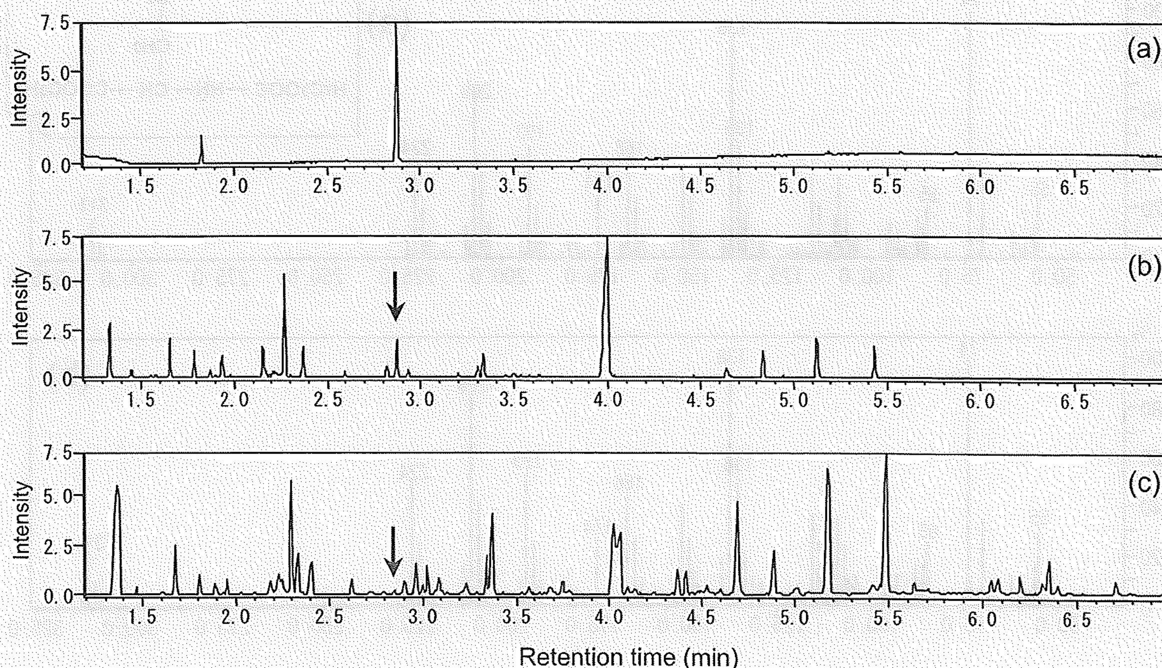

Fig. 1 Gas chromatpgrams of derivatized *Se*-methylselenocysteine (a), extact from selenium-enriched *Kaiware* radish sprouts (b) and extract from selenium-enriched garlic bulb (c).

のガスクロマトグラムを示したものである。Fig. 1(a)に示すように，標準 MeSec の誘導体に由来するピークは保持時間 2.87 分付近に認められた。これに対して，誘導体化処理したセレン強化カイワレダイコンスプラウト抽出液とセレン強化ニンニク抽出液のクロマトグラムにも，Fig. 1 (b) および (c) のように，標準 MeSec の誘導体と同じ保持時間を示す化合物の存在が認められた。

Fig. 2 は，Fig. 1(b)において保持時間 2.87 分を示した化合物のマススペクトルを標準 MeSec の誘導体のマススペクトルと比較したものである。両者のマススペクトルはほぼ一致していた。また，Fig. 1(c)において保持時間 2.87 分を示した化合物もほぼ同様のマススペクトルを示した（データ略）。

天然には^{80}Se をはじめとする様々なセレンの安定同位体が存在する。この中で^{78}Se，^{80}Se，^{82}Se の 3 つに着目すると，それらの天然における存在比（^{78}Se：^{80}Se：^{82}Se）は 2：4：1 に近似している。このことは，マススペクトルにおいて m−2，m，m+2 m/z の比が 2：4：1 を示す分子イオンピークやフラグメントイオンピークが存在すれば，その化合物がセレンを含有する可能性が高いことを意味する。本実験で用いたアミノ酸分析キットを用いると，Fig. 2 に記した構造式のように，アミノ酸のアミノ基がカルボキシプロピル化，カルボキシル基がプロピル化されるので，誘導体の分子量はもとのアミノ酸よりも 128 増加する。Fig. 2(a) および (b) には，誘導体化 MeSec（$C_{11}H_{21}O_4NSe$）の分子イオンに由来する 309，311，313 m/z，および誘導体からカルボキシプロピル基（C_3H_7COO-）が 1 つとれたフラグメントイオンに由来する 222，224，226 m/z がいずれも約 2：4：1 の比で認められる。また，他にも m−2，m，m+2 m/z の比が 2：4：1 となっているイオンピークがいくつか存在しており，これらは誘導体化MeSecのマススペクトルの大きな特徴といえる。以上のことから，Fig. 1 (b) および (c) で認められた保持時間 2.87 分を示す化合物は誘導体化 MeSec であり，セレン強化カイワレダイコンスプラウトとセレン強化ニンニク中に MeSec の存在することを GC-MS を用いて証明できたと考える。

2. セレン強化酵母

多くの研究によって，セレン強化酵母中のセレンの分子種のほとんどはセレノメチオニン（SeM）であることが明らかにされている[7]。そこで本実験では，セレン酵母中に存在すると考えられる SeM を誘導体化後，GC-MS を用いて同定することを試みた。

Fig. 3 は，EZ：faast™ を用いて誘導体化した SeM，および誘導体化処理したセレン強化酵母抽出液のガスクロマトグラムを示したものである。Fig. 3 (a) に示すように，標準 SeM の誘導体に由来するピークは保持時間 3.23 分付近に認められた。これに対して，セレン強化酵母抽出液のクロマトグラムにも，Fig. 3 (b) のように，標準 MeSec の誘導体と同じ保持時間を示す化合物が認められた。

Fig. 4 は，Fig. 3 (b) で認められた保持時間 3.23 分を示す化合物のマススペクトルを標準 SeM の誘導体と比較し

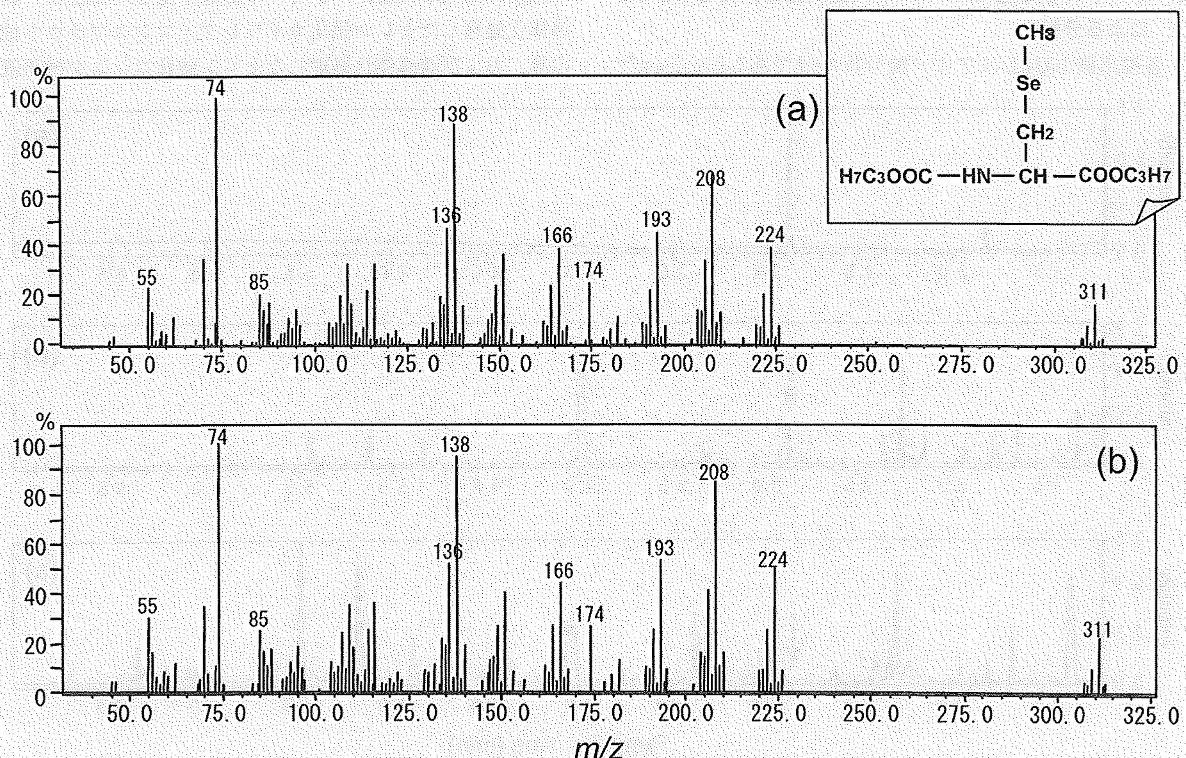

Fig. 2 Mass spectrums of derivatized *Se*-methylselenocysteine (a) and unknown compound contained in extract from selenium-enriched *Kaiware* radish sprouts (b).

たものである。両者のマススペクトルはほぼ一致していた。とくに，誘導体化 MeSec（$C_{12}H_{23}O_4NSe$）の分子イオンに由来する 323，325，327 *m/z*，フラグメント CH_3SeCH_2 に由来する 107，109，111 *m/z*，フラグメント $CH_3SeCH_2CH_2$ に由来する 121，123，125 *m/z* などのセレン化合物の特徴を示すイオンピークが共通して認められた。したがって，Fig. 3（b）で認められた保持時間 3.23 分の化合物は誘導体化 SeM であり，セレン強化酵母中に MeSec の存在することも GC-MS を用いて証明できたといえる。

3. セレン強化リョクトウスプラウト

セレン強化リョクトウスプラウト中のセレンの分子種については報告例が存在しない。そこでまず，HPLC-ICPMS を用いて，セレン強化リョクトウスプラウト中のセレンの分子種を推定した。セレン強化リョクトウスプラウト抽出液を HPLC-ICPMS で分析したところ，ほとんどのセレンは SeHL と同じ保持時間（5.1 分）に溶出され，セレン強化リョクトウスプラウトに SeHL の存在することが推察された（データ略）。次に，他の試料と同様に，標準 SeHL を誘導体化処理し，GC-MS で分析した。しかし，標準 SeHL の誘導体を検出することはできなかった。今回用いたアミノ酸分析用キット（EZ:faast™）は一般的なアミノ酸の中でアルギニンの分析ができない。これは本キットで誘導体化したアルギニンの沸点が高いためである。おそ

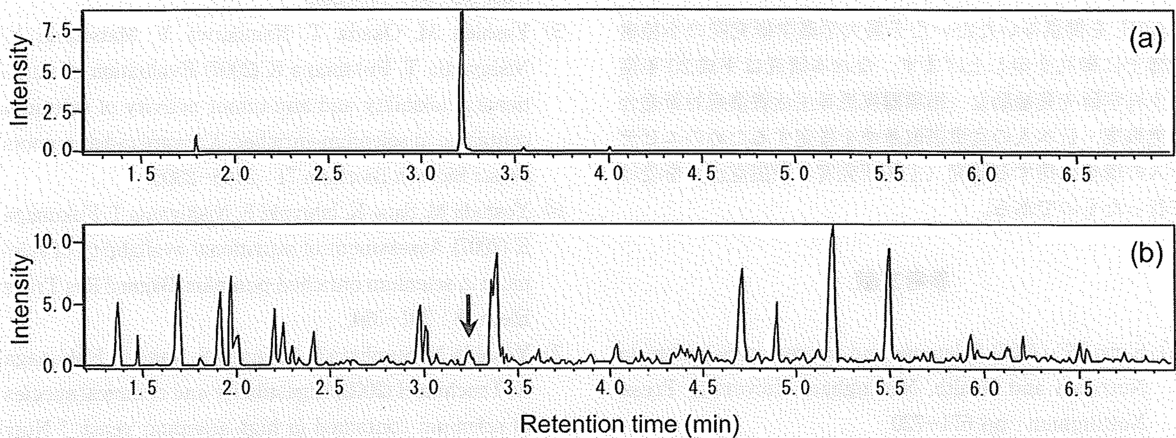

Fig. 3 Gas chromatograms of derivatized selenomethionine (a) and extract from selenium-enriched yeast (b).

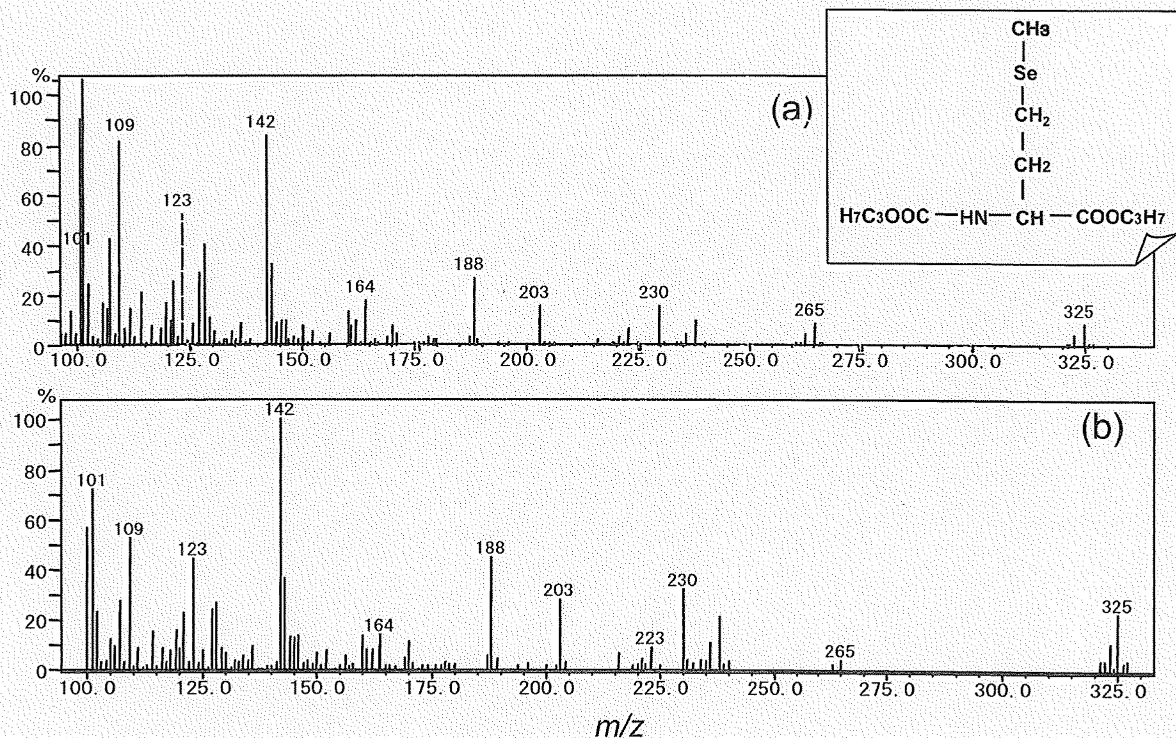

Fig. 4 Mass spectrums of derivatized selenomethionine (a) and unknown compound contained in extract from selenium-enriched yeast (b).

らくSeHLの誘導体もアルギニンの誘導体と同様に高沸点であるため，分析ができなかったと考えられる。

今回，GC-MS分析に用いたアミノ酸分析用キット（EZ：faast™）は，種々のアミノ酸を，誘導体化を含めて30分以内で定量分析できるようにしたものである。ただし，分析感度はあまり高くなく，含セレンアミノ酸を同定・定量するには，試料中セレン濃度が数ppm以上必要であった。したがって，一般の食品の含セレンアミノ酸の分析に用いる場合には，含セレンアミノ酸画分の濃縮操作が必要であり，さらに検討が必要と判断された。

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ORIGINAL ARTICLE

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Low plasma phylloquinone concentration is associated with high incidence of vertebral fracture in Japanese women

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Abstract It has been reported that vitamin K supplementation effectively prevents fractures and sustains bone mineral density in osteoporosis. However, there are only limited reported data concerning the association between vitamin K nutritional status and bone mineral density (BMD) or fractures in Japan. The objectives were to evaluate the association between plasma phylloquinone (K_1) or menaquinone (MK-4 and MK-7) concentration and BMD or fracture in Japanese women prospectively. A total of 379 healthy women aged 30–88 years (mean age, 63.0 years) were consecutively enrolled. Plasma K_1, MK-4, MK-7, and serum undercarboxylated osteocalcin (ucOC) concentrations, BMD, and incidence of vertebral fractures were evaluated. In stepwise multiple linear regression analyses, L_{2-4} BMD and a bone turnover marker, log K_1, concentrations were independently correlated with vertebral fracture incidence. When subjects were divided into low and high K_1 groups by plasma K_1 concentration, the incidence of vertebral fracture in the low K_1 group (14.4%) was significantly higher than that in the high K_1 group (4.2%), and its age-adjusted RR was 3.58 (95% CI, 3.26–3.93). L_{2-4} BMD was not different between the two groups. These results suggest that subjects with vitamin K_1 insufficiency in bone have increased susceptibility for vertebral fracture independently from BMD.

Key words vitamin K · undercarboxylated osteocalcin · vertebral fracture · bone mineral density (BMD) · Japanese women · phylloquinone

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Introduction

Vitamin K is well known for its role in the synthesis of a number of blood coagulation factors. Vitamin K is also an important factor for bone metabolism via γ-carboxylation of vitamin K-dependent proteins such as osteocalcin (OC), matrix Gla protein, and protein S [1,2]. Low dietary phylloquinone (K_1) intake has been shown to be associated with increased hip fracture risk, notably among postmenopausal women [3,4]. Low dietary K_1 intake is also associated with low bone mineral density (BMD) at the hip and spine in pre- and postmenopausal women [5,6], and circulating levels of vitamin K_1 or K_2 were reported to be decreased in patients with hip fracture [7–10]. Those studies were mainly performed in Caucasians. There is only a limited amount of data concerning the association between vitamin K nutritional status and BMD or fractures in Japan. It has been reported that the intake of *natto*, which contains a high concentration of menaquinone-7 (MK-7), prevents hip fractures in Japanese [11] or promotes bone formation in premenopausal women [12]. However, another report showed that no differences in plasma K_1, menaquinone-4 (MK-4), and MK-7 were observed between patients with vertebral or hip fracture and normal subjects [13]. In animal models of osteoporosis, the effects of vitamin K_2 supplementation on bone mass, strength, and structure has been reported to be effective [14–17], or to be negative in ovariectomized rats [18–20], and the evidence is still equivocal. Although a relationship between vitamin K status and fracture risk has been reported, the relationship between BMD or fracture and vitamin K status is still controversial. Recently, it has been reported that vitamin K stimulates the differentiation of osteoblasts via not only γ-carboxylation but also steroid or xenobiotics receptors (SXR) [21].

Therefore, in the present study, we evaluated the association between plasma vitamin K (K_1, MK-4, and MK-7) concentrations and incidence of fracture or BMD in Japanese women prospectively, and assessed the importance of vitamin K status or γ-carboxylation of OC in reduction of fracture risk and increase of BMD.

Subjects and methods

Subjects

Japanese women in their thirties to eighties were consecutively enrolled in this study (2002–2003), and followed up by 2006. Women with metabolic bone diseases other than primary osteoporosis and women who were taking medicine related to bone metabolism such as active vitamin D, vitamin K, vitamin K antagonists, estrogen, bisphosphonates, or steroids were excluded. Women who had extremely low body mass index (BMI) (lower than 16) were also excluded. A total of 379 women (mean age, 63.0 ± 10.8 years; range, from 30 to 88 years) met the selection criteria for this study. The subjects consisted of 48 women aged 30–49 years, 202 women aged 50–69 years, and 129 women aged 70 years or older (70– years). Subjects were living in a rural area of Nagano. Most subjects have a backyard with their house, and they had the habit of frequently eating vegetables that they cultivated in their backyard.

Measurements

Plasma, serum and urine samples were collected from the subjects in the morning and stored immediately at −30°C until measurement. Plasma vitamin K (K_1, MK-4, and MK-7) was determined by the high-performance liquid chromatography-tandem mass spectrometry (LC-APCI-MS/MS) method [22]. ucOC as a sensitive marker for vitamin K insufficiency was measured by electrochemiluminesence immunoassay (ECLIA) (Sanko Junyaku, Japan). The antibody used in this ECLIA method is the same antibody used in the "Takara assay." However, the ucOC concentrations measured using this novel method were higher than those obtained using other methods, including the Takara assay. Intact OC was determined by immunoradiometric assay (IRMA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Japan).

Serum concentrations of 25-hydroxyvitamin D [25-OH-D; radioimmunoassay (RIA); DiaSorin, Stillwater, MN, USA], and intact (1-84, 7-84) parathyroid hormone [intact PTH, immunoradiometric assay (IRMA); Scantibodies Laboratory, Santee, CA, USA] were determined. A bone resorption marker, urinary excretion of *N*-telopeptide (NTX; as measured by enzyme-linked immunosorbent assay (ELISA; Osteomark, Ostex International Seattle, WA, USA), and a bone formation marker, bone-derived alkaline phosphatase (BAP; EIA; DS Pharma Biomedical, Japan), were measured. For the evaluation of calcium metabolism, serum concentrations of calcium (Ca) and phosphorus (P) were measured. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

Lumbar spine (L_{2-4}) and femoral neck (FN) BMD was measured by dual-energy X-ray absorptiometry (DXA) using a Lunar DPX-IQ (Lunar, Radisson, WI, USA). The interassay variance of this method in our laboratory was $0.5\% \pm 0.5\%$ [coefficient of variation (CV) ± SD] [23]. Incident vertebral fracture was first defined by the semiquantitative method reported by Genant et al. [24]. When a marginal fracture was obtained, we performed quantitative measurements of vertebral body heights at the posterior, central, and anterior margins in both baseline and follow-up vertebral films. We then redefined the presence or absence of incident vertebral fractures in accordance with the criteria proposed by Fukunaga et al. [25]. Fractures were evaluated by one of the coauthors who had contributed to development of the method of Fukunaga et al. [25]. Incident fractures with apparent major trauma were excluded from the present study because we wanted to examine the relationship between vitamin K nutrition and fragility fracture occurrence.

Statistical analysis

All statistical analyses were performed by using statistical software JMP 6.0 J (SAS Institute, Cary, NC, USA). Logistic regression analysis was used to test univariable associations between the incidence of vertebral fracture and anthropometric parameters, bone metabolic parameters, or plasma vitamin K concentrations. Stepwise multiple linear regression analyses were performed to explore determinants of incident vertebral fractures. The following plausible predictors were included in the original model: (1) age, BAP, and K_1 concentration, and (2) L_{2-4} BMD, BAP, and K_1 concentration. Variables that correlated strongly with each other, such as age and L_{2-4} BMD, were not entered simultaneously in the original model. Forward stepwise regression was performed, and $P < 0.25$ was used to enter variables. Values of vitamin K concentrations were logarithmically transformed to improve normality in this analysis because plasma vitamin K concentrations were not normally distributed. A Cox proportional hazards model was used to assess the relationship between plasma K_1 concentration and vertebral fracture. Hazard ratios and 95% confidence intervals are evaluated by no adjusted model or adjusted model for BMD or BMI.

In the second analysis, subjects were divided into low and high K_1 groups by median K_1 concentration (2.67 nmol/l). Parametric comparisons used Student's *t* test. The incidence of vertebral fracture in the two groups was evaluated by the chi square test and crude or age-adjusted relative risks (RRs). Moreover, the age and L_{2-4} BMD values at which 25% of subjects would suffer fractures in the four groups were inversely predicted by logistic regression analysis.

Ethical considerations

The comprehensive study protocol including nutritional evaluation was reviewed by the ethics committee of Research Institute and Practice for Involutional Diseases (RIPID), and comprehensive written informed consent was obtained from all participants.

Table 1. Subject characteristics

n	379
Age (years)	63.0 (10.8)
Body weight (kg)	52.1 (7.3)
Body height (cm)	151.6 (6.0)
BMI (kg/m^2)	22.6 (2.8)
K_1 (nmol/l)	3.51 (2.70)
MK-4 (nmol/l)	0.20 (0.31)
MK-7 (nmol/l)	10.0 (15.1)
ucOC (ng/ml)	4.68 (3.15)
iOC (ng/ml)	8.69 (7.13)
25-OH-D (nmol/l)	51.8 (16.3)
iPTH (pmol/l)	4.9 (1.8)
Ca (mmol/l)	2.30 (0.10)
P (mmol/l)	1.12 (0.15)
BAP (U/l)	31.4 (11.2)
NTX (pmol BCE/μmol Cr)	57.3 (25.5)
$L_{2\text{–}4}$ BMD (g/cm^2)	0.970 (0.186)
$L_{2\text{–}4}$ Z-score	0.178 (1.405)
FN BMD (g/cm^2)[a]	0.750 (0.128)
FN BMD Z-score[a]	0.398 (0.857)

All values are mean (SD)
K_1, phylloquinone; MK, menaquinone; ucOC, undercarboxylated osteocalcin; iOC, intact osteocalcin; 25-OH-D, 25-hydroxyvitamin D; iPTH, intact parathyroid hormone, BAP, bone-derived alkaline phosphatase; NTX, N-terminal telopeptide; BCE, bone collagen equivalent; BMD, bone mineral density; $L_{2\text{–}4}$, lumbar spine$_{2\text{–}4}$; FN, femoral neck
[a]FN BMD and FN BMD Z-score were measured in 176 subjects
Plasma and urinary biochemical parameters were within the normal range

Results

Subject characteristics

The subject characteristics are summarized in Table 1. The plasma K_1, MK-4, and MK-7 concentrations (mean ± SD) of the 379 Japanese women were 3.51 ± 2.70, 0.20 ± 0.32, and 10.0 ± 15.1 nmol/l, respectively. Other plasma and urinary biochemical parameters were within the normal range. The location and number of incident fracture were as follows: vertebrae, 35 (9.2%); forearm, 8 (2.1%); femoral neck, 1 (0.3%); and others, 5 (1.3%). Because there were few cases of forearm and femoral neck fractures, the incidence of vertebral fracture was used to evaluate the association between vitamin K status and bone fracture.

Association between plasma vitamin K concentration and incidence of vertebral fracture

Table 2 shows the association between the incidence of vertebral fracture and age, anthropometric parameters, bone metabolic parameters, and plasma vitamin K concentrations. Age ($P < 0.001$) and BAP ($P = 0.011$) were associated positively, and $L_{2\text{–}4}$ BMD ($P < 0.001$), K_1 ($P = 0.007$), and log K_1 ($P < 0.001$) were associated negatively with the incidence of vertebral fracture. MK-4 and MK-7 concentrations were not associated with the incidence of vertebral fracture. NTX and log ucOC showed a tendency to be positively associated with the incidence of vertebral fracture, and their P values were almost equal (NTX, $P = 0.089$; log ucOC, $P = 0.088$).

Table 2. Association between incidence of vertebral fracture and age, anthropometric parameters, bone metabolic parameters, and plasma vitamin K concentrations

	β-Coefficient	P
Age (years)	0.064	<0.001
BW (kg)	0.028	0.240
BH (cm)	−0.032	0.274
$L_{2\text{–}4}$ BMD (g/cm^2)	−3.956	<0.001
NTX (pmol BCE/μmol Cr)	0.012	0.089
BAP (U/l)	0.042	0.011
ucOC (ng/ml)	0.057	0.271
ucOC/iOC	0.145	0.698
Log ucOC	0.487	0.088
Log ucOC/iOC	0.213	0.518
K_1 (nmol/l)	−0.244	0.007
MK-4 (nmol/l)	−0.345	0.602
MK-7 (nmol/l)	−0.005	0.672
Log K_1 (nmol/l)	−0.899	<0.001
Log MK-7 (nmol/l)	−0.057	0.672

Logistic regression analysis was used to test univariate associations of anthropometric or bone metabolic parameters and plasma vitamin K concentrations with incidence of vertebral fracture
Age and BAP were associated positively, and L2–4 BMD, K1, and log K1 were associated negatively with vertebral fracture incidence

Table 3. Relationship between vertebral fracture incidence and age, $L_{2\text{–}4}$ BMD, BAP, or plasma vitamin K_1 concentration evaluated by stepwise multiple regression analysis

a. Plausible predictors (age, BAP and log K_1)

	Estimate	r^2	P
Age	0.050	0.055	0.017
Log K_1	−0.783	0.033	0.014
BAP	0.040	0.029	0.017

b. Plausible predictors ($L_{2\text{–}4}$ BMD, BAP, and log K_1)

	Estimate	r^2	P
$L_{2\text{–}4}$ BMD	−4.125	0.096	0.001
Log K_1	−0.760	0.033	0.017
BAP	0.036	0.022	0.039

Stepwise multiple linear regression analyses were performed to identify determinants of vertebral fracture incidence
The following plausible predictors were included in the original model: (1) age, BAP, and vitamin K_1 concentration (log K_1), (2) $L_{2\text{–}4}$ BMD, BAP, and vitamin K_1 concentration (log K_1)
Variables that correlated strongly with each other, such as age and $L_{2\text{–}4}$ BMD, were not entered simultaneously into the original model
Age, $L_{2\text{–}4}$ BMD, BAP, and log K_1 concentration were independently associated with vertebral fracture incidence

Stepwise multiple linear regression analyses were performed to explore the determinants of vertebral fracture incidence. In both models, (1) age, BAP, and log K_1 and (2) $L_{2\text{–}4}$ BMD, BAP, and log K_1were included in the original model, and age, $L_{2\text{–}4}$, BMD, BAP, and log K_1 concentration were independently associated with the incidence of vertebral fracture (Table 3). Moreover, a Cox proportional hazards model was used to assess the relationship between plasma K_1 concentration and vertebral fracture (Table 4). Hazard ratios and 95% confidence intervals are evaluated by no adjusted model or adjusted model for BMD or BMI. Both plasma K_1 concentration and log K_1 concentration

significantly decreased hazard ratio of vertebral fracture in the no adjusted model and adjusted model for BMD or BMI. Significant association between vitamin K_1 concentration and vertebral fracture was not observed in the age-adjusted model, because age and vitamin K_1 concentration became a strong confounding factor in the Cox proportional hazards model including a time course factor

Vertebral fracture incidence in low and high K_1 groups

Comparison of the incidence of vertebral fracture between the low and high K_1 groups was divided by the median plasma K_1 concentration (2.67 nmol/l) (Table 5). The incidence of vertebral fracture in the low K_1 group (n = 27, 14.4%) was significantly higher than that in the high K_1 group (n = 8, 4.2%), $P < 0.001$. The age of the low K_1 group was significantly higher than that of the high K_1 group. However, no significant difference was observed in L_{2-4} BMD between the two groups. The unadjusted RR for vertebral fractures in the low K_1 group was 3.43 [95% confidence interval (CI), 1.60–7.35) and the age-adjusted RR was 3.58 (95% CI, 3.26–3.93). No significant differences of plasma 25-OH-D (low K_1, 52.8 ± 17.3; high K_1, 51.0 ± 15.3 nmol/l) or PTH (low K_1, 3.3 ± 1.4; high K_1, 3.3 ± 1.2 pmol/l) concentrations were observed between the two groups. Moreover, the inverse prediction values of L_{2-4} BMD at which 25% of subjects would suffer fractures were estimated from logistic regression analysis in the two groups. The predicted L_{2-4} BMD in the low K_1 group was 0.707 (95% CI, 0.053–0.847, P = 0.007), and that in the high K_1 group was 0.578 (95% CI, 0.004–0.711, P = 0.003). These results suggest that subjects with low vitamin K status would suffer fractures at a higher BMD than those with high vitamin K status.

Table 4. Hazard ratio (HR) of vertebral fracture evaluated by Cox proportional hazards model

Variables	HR	95% CI	P	Adjustment
K_1	0.628	0.404–0.899	0.008	No
	0.691	0.453–0.982	0.038	BMD
	0.656	0.415–0.940	0.018	BMI
Log K_1	0.561	0.363–0.867	0.009	No
	0.612	0.397–0.948	0.028	BMD
	0.517	0.332–0.808	0.004	BMI

A Cox proportional hazards model was used to assess the relationship between plasma K_1 concentration and vertebral fracture; hazard ratios (HR) and 95% confidence intervals (CI) are evaluated by no adjusted model or adjusted model for BMD or body mass index (BMI)

Discussion

The associations between dietary vitamin K intake, biochemical indicators of vitamin K status such as plasma K_1 or ucOC concentration, and bone loss and risk of hip fracture were evaluated in several studies [3–10,25]. Low dietary K_1 intake has been reported to be associated with increased hip fracture risk, most notably in postmenopausal women [3,4]. In the Framingham Heart Study, low dietary K_1 intake was not associated with low BMD at either the hip or spine, even though low intake was associated with increased hip fracture risk [3]. However, in the Framingham Heart Study (1996–2000) [6], low plasma K_1 concentration after adjustment for plasma triglyceride concentration was associated with low BMD at the femoral neck among the men and low plasma K_1 concentration was associated with low spine BMD in postmenopausal women. In other studies, low dietary K_1 intake was associated with low BMD in women aged 29–86 years [5], and low plasma K_1 concentration was shown to be associated with low BMD at the spine [26]. The vitamin K concentration in elderly women with hip fractures was reported to be low [7–10]. Although an apparent relationship between vitamin K status and fracture risk has been reported, the relationship between BMD and vitamin K status is still controversial. Therefore, the mechanism(s) responsible for reducing fracture risk with high vitamin K intake or high serum level of vitamin K are not fully understood.

In the present study, the associations between plasma K_1, MK-4, and MK-7 concentrations and incidence of fracture were evaluated in Japanese women. The results showed a significant association between plasma K_1 concentration and incidence of vertebral fracture. Moreover, we could demonstrate that K_1 concentration was associated with vertebral fracture incidence independently of age, L_{2-4} BMD, and BAP. However, vitamin K status and femoral neck or other fractures could not be evaluated in the present population because of the lack of statistical power of these long

Table 5. Relative risk of vertebral fracture incidence in two groups divided by plasma vitamin K_1 concentration

Groups	n	Age	BMD	BAP	Incidence of vertebral fracture	RR (95% CI)	Age-adjusted RR (95% CI)
Low K_1	188	65.3 (12.1)	0.966 (0.195)	31.0 (11.7)	14.4%	3.43 (1.60–7.35)	3.58 (3.26–3.93)
High K_1	191	62.7 (10.1)	0.973 (0.177)	31.8 (10.7)	4.2%	1	1
P		0.020	0.708	0.478	<0.001		

Mean (SD)
Subjects were divided into two groups according to the median of plasma K_1 concentration (2.67 nmol/l)
Student's t test was used to compare the age of the two groups
Crude and age-adjusted relative risks (RRs) for the vertebral fracture incidence are presented with 95% confidence intervals
Crude and age-adjusted RRs for vertebral fracture incidence of the low K_1 group were significantly higher than those of the high K_1 group

bone fractures. In the present study, the numbers of incident femoral neck and forearm fractures were 1 and 8, respectively. A lower prevalence of hip fracture in the Japanese population than in Caucasians was reported [27]. Thus, evaluation of the role of vitamin K in long bone fracture in the Japanese population will require a larger sample size.

In a previous study, it was shown that high serum MK-7 concentration resulting from eating *natto*, which is a high-MK-7-content food, may contribute to the relatively low hip fracture risk in Japanese women [11]. However, in the present study, we did not find that plasma MK-7 concentration was associated with vertebral fracture incidence. It has been reported that MK-7 has equivalent potency regarding γ-carboxylation of OC to K_1 [28–30] and that *natto* intake promotes bone formation in premenopausal woman [12]. The reason why the association between MK-7 concentration and vertebral fracture was weaker than the associations between K_1 concentration and vertebral fracture is not clear. In a previous study [12], the association between the prevalence of femoral neck fracture and the consumption of *natto* was evaluated by comparison of the rate of the fracture between areas with and without the custom of eating *natto*. However, almost all subjects were *natto* eaters in the present study, which may be one of the reasons why no significant association between plasma MK-7 concentration and vertebral fracture incidence was observed. Moreover, a survey of the period or interval of MK-7-rich food intake seems more important than the measurement of serum MK-7 concentration for evaluating the relationship between bone metabolism and MK-7 in Japan. However, unfortunately, a food questionnaire was not employed in the present study, and this will be necessary in future.

Not only the circulating K_1 concentration but also the serum ucOC concentration has been reported to be associated with hip fracture [31–34]. We have reported that circulating K_1 and MK-7 concentrations were negatively correlated with the serum ucOC concentration; however, the level of vitamin K_1 or MK-7 required to reduce the serum ucOC concentration increased with advanced age [35]. In the present study, ucOC concentration or the ratio of ucOC/intactOC did not show a significant association with incident vertebral fracture. Recent studies revealed that vitamin K may play two important roles in bone metabolism, one of which is regulating posttranslational modification of Gla-containing proteins, and the other is regulating the SXR-mediated cellular regulatory system. Recently, Ichikawa et al. [36] reported that collagen accumulation in osteoblastic MG63 cells was enhanced by vitamin K_2 treatment, and the transcription of the extracellular matrix-related gene "*tsukushi*," which is involved in collagen assembly, was regulated by vitamin K_2 via steroid and xenobiotic receptor (SXR). Therefore, vitamin K plays a significant role in bone homeostasis, not only by affecting γ-carboxylation but also by affecting transcriptional regulation of the collagen gene, which may be one of the reasons why the association between ucOC and fracture incidence was weak as compared with that between K_1 and fracture.

In the second analysis, subjects were divided into low and high K_1 groups according to median K_1 concentration (2.67 nmol/l). The low K_1 group showed a higher incidence of vertebral fracture (Table 5). The age of the low K_1 group was also higher than that of the high K_1 group (Table 5). However, both the unadjusted and age-adjusted RRs demonstrated that risk of vertebral fracture was greater in the low K_1 status group. Moreover, L_{2-4} BMD was not different between the two groups, suggesting that K_1 status may be associated with vertebral bone strength, not with L_{2-4} BMD. The inverse predicted value of L_{2-4} BMD at which 25% of the subjects would suffer fractures was significantly higher in the low K_1 group. This finding suggests that subjects with low vitamin K_1 status would easily suffer fractures even with higher L_{2-4} BMD.

In the present study, the average of K_1 concentration was 3.51 nmol/l, and it was two or three times higher than previous reports. Averages of circulating K_1 concentrations in European or U.S. subjects have been reported approximately within the range of 0.7 to 1.7 nmol/l [6,10,37–42]. In other reports of Japanese subjects, 1.58 [26], 1.07 [13], 1.86 [43], and 2.66 [44] nmol/l K_1 concentrations were reported. Average of K_1 concentration in our other epidemiological study of Japanese elderly subjects was 1.71 nmol/l (data have not been published). Precision and accuracy of LC-APCI-MS/MS method used in present study to measure the vitamin K concentration had been confirmed by the HPLC fluorescence determination method [45]. Correlation coefficient and the corresponding P value for K_1 concentration determined by LC-APCI-MS/MS and HPLC fluorescence determination methods were $r = 0.989$ and $P < 0.001$ ($y = 0.841\,x + 0.035$ ng/ml; y, HPLC fluorescence determination method; x, LC-APCI-MS/MS method). From these results, the circulating K_1 concentration of Japanese subjects is considered to be higher than that of European or U.S. subjects, and dietary K_1 intake of Japanese people suggests that the K_1 intake in Japanese may be higher than that in Europe countries of the United States. The reason why the average K_1 concentration in the present study was particularly higher than other studies not only in Europe and the United States but also in Japan was not clear. Subjects were living in a rural area of Nagano. Most subjects have a backyard at their house, and they have the habit of frequently eating the vegetables that they cultivate in their backyard. Thus, although a food questionnaire was not employed in the present study, it is predicted that the dietary K_1 intake of present subjects may be relatively high.

There were some limitations of the present study. The design was a prospective study, but the participants were recruited from a hospital in a rural area of Japan (refer to the paper by Shiraki et al. [46] for the characteristics of this population). Thus, a nationwide prospective survey is required to assess the role of vitamin K in bone fractures conclusively in the near future. Although there were some limitations of the present study, it can be concluded that the incidence of vertebral fractures was associated with the plasma K_1 concentration. Because subjects with low vitamin K_1 status showed increased risk of vertebral fractures

regardless of their L_{2-4} BMD, low vitamin K_1 status may be an indicator of low bone quality.

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Vitamin D status, bone mass, and bone metabolism in home-dwelling postmenopausal Japanese women: Yokogoshi Study

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Abstract

Little has been understood about vitamin D status in relation to bone health in Asian women. The purpose of this study was to identify how the serum 25-hydroxyvitamin D (25[OH]D) concentration is associated with bone mass and bone metabolism. This cross-sectional, community-based epidemiologic study was conducted among 600 ambulatory postmenopausal women. The serum 25(OH)D concentration was measured with radioimmunoassay. Other blood biochemical measurements were intact parathyroid hormone and markers of bone turnover, including osteocalcin and type I collagen cross-linked N-telopeptides. Bone mineral density (BMD) of the lumbar spine and right femoral neck were measured with the dual-energy X-ray absorptiometry method using a QDR4500a. The mean serum 25(OH)D concentration was 55.6 nmol/L (SD 14.6). Serum 25(OH)D concentration was linearly associated with BMD of the femoral neck (R^2=0.020, P=0.003), but not with BMD of the lumbar spine. Odds ratios (ORs) for low BMD (defined as t score ≤−2.5 SD) were calculated for strata defined by 25(OH)D concentration. The prevalence of low BMD of the lumbar spine was significantly higher in the 40- to 50-nmol/L 25(OH)D group (adjusted OR=3.0, 95% CI: 1.3–7.0) compared to the reference group (≥70 nmol/L). Prevalence of low BMD for the femoral neck was significantly higher in the 30- to 40-nmol/L (adjusted OR=3.6, 95% CI: 1.1–12.1) and the 40- to 50-nmol/L (adjusted OR=7.6, 95% CI: 2.5–23.2) groups compared to the reference group (≥70 nmol/L). The mean serum concentration of intact PTH was significantly higher in subjects with serum 25(OH)D <50 nmol/L compared to those with serum 25(OH)D ≥50 nmol/L. The present study suggests that higher serum 25(OH)D concentrations are associated with increased BMD of the femoral neck, and that a serum 25(OH)D concentration of at least 70 nmol/L is needed to obtain high BMD of the femoral neck, and that of at least 50 nmol/L is needed to achieve normal PTH levels and prevent low BMD in home-dwelling postmenopausal Japanese women.
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Introduction

Vitamin D insufficiency is an important risk factor for the development of osteoporosis and osteoporotic fractures in the elderly. One mechanism by which this excess risk is conferred is through an increase in parathyroid hormone production [1]. Moreover, vitamin D insufficiency may cause decreased muscle function and standing balance [2], leading to an increased frequency of falls. Supplementation with vitamin D, particularly among the elderly and among women, is recommended in many European and North American countries.

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