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[8,9]。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。

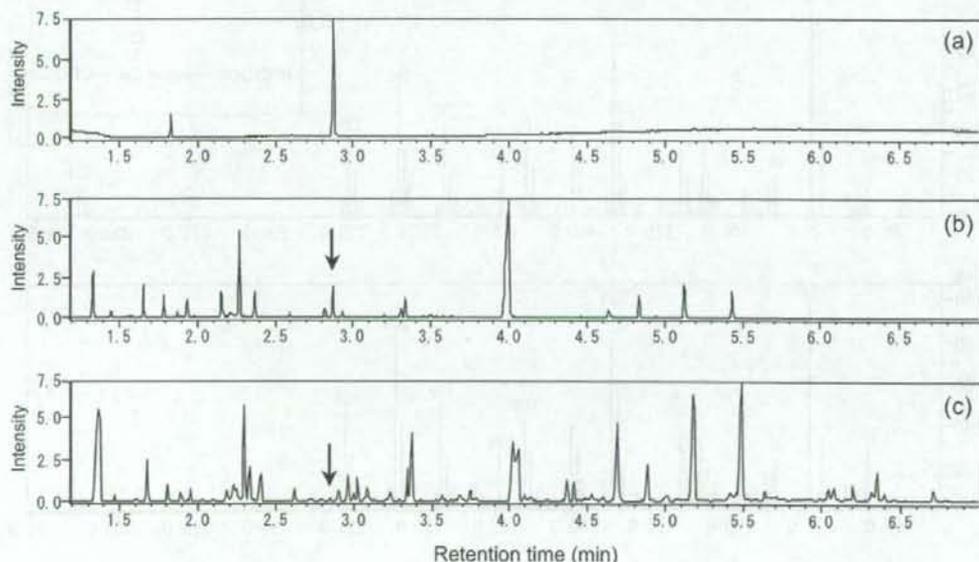Fig. 1は、EZ：faast™を用いて誘導体化したMeSec、誘導体化処理したセレン強化カイワレダイコンスプラウト抽出液、および誘導体化処理したセレン強化ニンニク抽出液

Fig. 1　Gas chromatpgrams of derivatized Se-methylselenocysteine (a), extract 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。

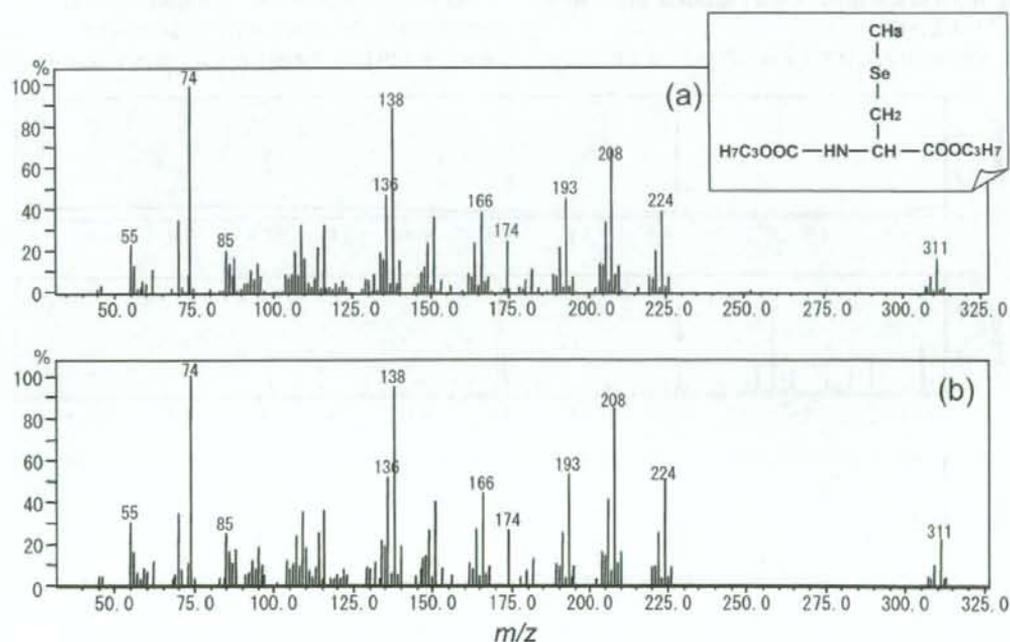Fig.4は、Fig.3(b)で認められた保持時間 3.23 分を示す化合物のマススペクトルを標準 SeM の誘導体と比較し

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（$C_{12}H_{25}O_4NSe$）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

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
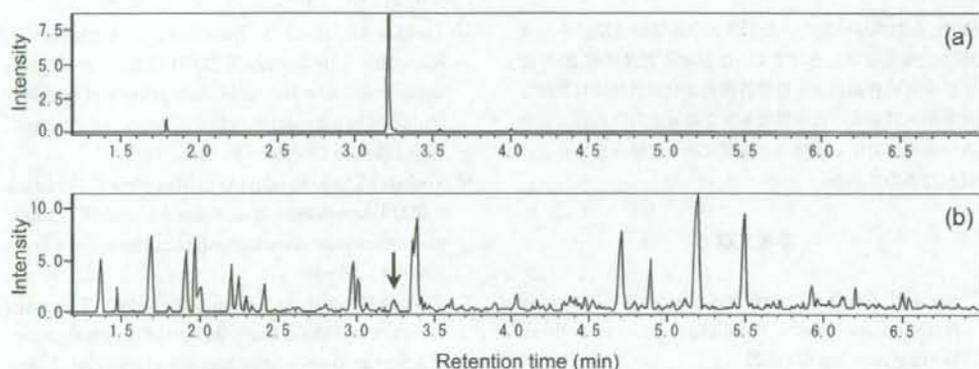

Fig. 3 Gas chromatograms of derivatized selenomethionine (a) and extract from selenium-enriched yeast (b).
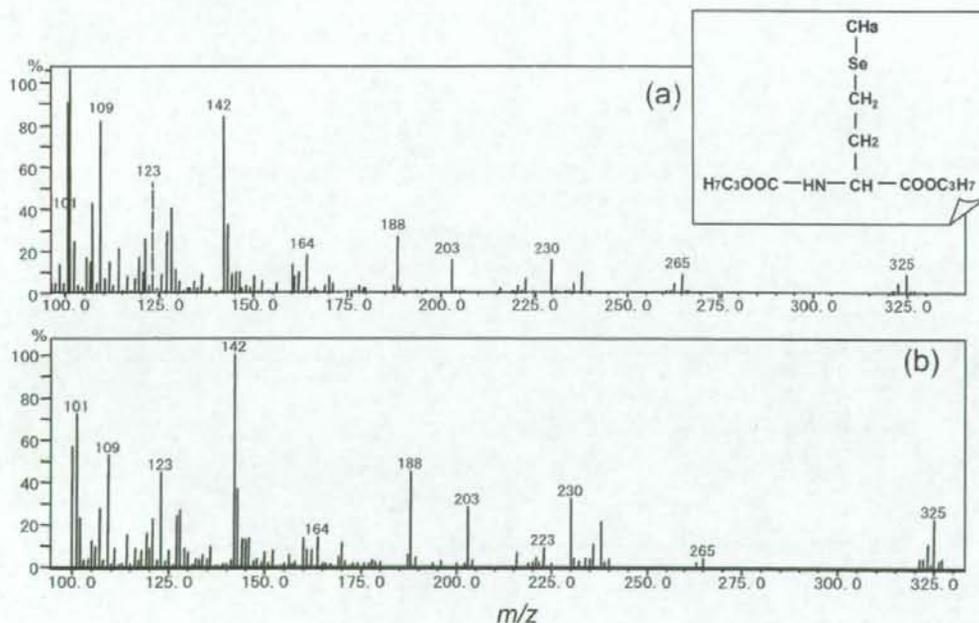

Fig. 4 Mass spectrums of derivatized selenomethionine (a) and unknown compound contained in extract from selenium-enriched yeast (b).

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。

謝　辞

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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

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) $\pm$ SD] [23]. Inci-

dent 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 6.0J (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$_{2-4}$ BMD (g/cm^2)	0.970 (0.186)
L$_{2-4}$ 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$_{2-4}$, lumbar spine$_{2-4}$; 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$_{2-4}$ 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$_{2-4}$ 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$_{2-4}$ 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$_{2-4}$ BMD, BAP, and log K$_1$)

	Estimate	r^2	P
L$_{2-4}$ 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$_{2-4}$ BMD, BAP, and vitamin K$_1$ concentration (log K$_1$)
Variables that correlated strongly with each other, such as age and L$_{2-4}$ BMD, were not entered simultaneously into the original model
Age, L$_{2-4}$ 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$_{2-4}$ BMD, BAP, and log K$_1$ were included in the original model, and age, L$_{2-4}$ 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.

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 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)

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.

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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Table 1

Demographic and physical characteristics, nutritional intakes, bone mineral density (BMD), and serum biochemical profiles of study subjects

	N	Mean	SD
Age (years)	600	63.5	5.8
Menopausal age (years)	598	51.0	8.3
Number of children	599	2.3	0.8
Height (cm)	600	150.7	5.5
Weight (kg)	600	53.1	8.3
Body mass index (kg/m^2)	600	23.4	3.5
Thigh muscle strength (kg)	584	36.0	7.6
Grip strength (kg)	599	23.2	3.9
Timed Up & Go test (s)	593	5.0	1.0
Calcium intake[a] (mg/day)	600	527	160
Vitamin D intake (µg/day)	600	11.7	2.7
BMD at lumbar spine (g/cm^2)	599	0.846	0.147
BMD at femoral neck (g/cm^2)	598	0.668	0.094
Serum 25-hydroxyvitamin D (nmol/L)	600	55.6	14.6
Serum 1,25-dihydroxyvitamin D (pmol/L)	598	130.5	44.5
Serum intact parathyroid hormone (pmol/L)	600	4.24	1.40
Serum osteocalcin (mg/ml)	600	9.93	3.95
Serum type I collagen cross-linked N-telopeptides (nmol BCE/L)	595	21.0	6.5

[a] Calcium intake from dietary source was 518 mg (SD 147).

There may be ethnic differences in the effects of low vitamin D status on bone mass or bone metabolism. African Americans typically have lower vitamin D levels than Caucasian Americans, yet they have a lower prevalence of osteoporosis [3]. Furthermore, the relationship between serum 25-hydroxyvitamin D (25[OH]D, an index of vitamin D status) concentrations and bone mineral density (BMD) may differ between blacks and whites [4]. These findings demonstrate the importance of studies aimed at understanding the effect of vitamin D status on bone in non-white populations.

There have been only a few studies on the association between vitamin D status and bone parameters in Asians; those that have been conducted have typically had small sample sizes. One large population-based study among Japanese elderly women reported a cutoff level of serum 25(OH)D concentration in relation to elevated serum parathyroid hormone (PTH) as low as 40 nmol/L. This is lower than cutoff levels reported recently by several studies among Caucasian patients [5] and is less than current recommended levels (75–80 nmol/L or higher) of serum 25(OH)D [6]. These results suggested a possible ethnic difference between Asians and whites.

The primary aim of this study is to investigate the association between the serum 25(OH)D concentration and bone mass or bone metabolism among Japanese postmenopausal women. Results from this study may inform the appropriate levels of serum 25(OH)D to aim for in preventive vitamin D supplementation programs for these women.

Subjects and methods

Subjects

All 1310 women who lived in Yokogoshi area (Niigata City, Japan) aged between 55 and 74 years on March 31, 2006, were invited to participate in the Yokogoshi Study, a cross-sectional, epidemiologic, community-based investi-

gation of bone health for postmenopausal women. The study was conducted in November 2005. Of the 1310 women, 674 (51.5%) agreed to participate in the study. All participants were non-institutionalized and ambulatory. The following women who had medical histories that may have affected their bone metabolism were excluded from analysis: (1) 13 women with a history of bilateral oophorectomy, (2) 7 women who had undergone corticosteroid therapy, and (3) 54 women treated with bisphosphonates, selective estrogen receptor modulators, active vitamin D analogues, vitamin K (menatetrenone), estrogen, or calcitonin for suspected osteoporosis. Ultimately, 600 of 674 (89%) women agreeing to participate in the study formed the group analyzed. Written informed consent was obtained from all subjects. The protocol of this study was approved by the Ethics Committee of Niigata University School of Medicine.

BMD measurement

BMDs of the lumbar spine (L2–4) and right femoral neck were measured through the dual-energy X-ray absorptiometry (DXA) method using a

Table 2

Results of simple linear regression analyses with bone mineral density (BMD) as the dependent variable

Predictor variable	BMD of the lumbar spine			BMD of the femoral neck		
	Regression coefficient (β)	R^2	P value	Regression coefficient (β)	R^2	P value
Age (years)	−0.00611	0.057	<0.0001	−0.00473	0.084	<0.0001
Years since menopause	−0.00289	0.039	<0.0001	−0.00193	0.042	<0.0001
Number of children	−0.00158	0.000	0.8312	−0.00216	0.000	0.6519
Height (cm)	0.00481	0.033	<0.0001	0.00322	0.036	<0.0001
Weight (kg)	0.00610	0.119	<0.0001	0.00440	0.151	<0.0001
Body mass index (kg/m^2)	0.0118	0.078	<0.0001	0.00854	0.099	<0.0001
Thigh muscle strength (kg)	0.00464	0.058	<0.0001	0.00341	0.076	<0.0001
Grip strength (kg)	0.00914	0.059	<0.0001	0.00541	0.050	<0.0001
TUG test[a] (s)	−0.101	0.013	0.0050	−0.0876	0.024	0.0002
Engage in housework (No, 0; yes, 1)	−0.00392	0.000	0.8908	−0.0179	0.002	0.3289
Engage in light exercise (No, 0; yes, 1)	−0.0110	0.001	0.3705	−0.00537	0.001	0.4936
Engage in farmwork (No, 0; yes, 1)	−0.0159	0.003	0.1864	−0.00164	0.000	0.8323
Calcium intake (mg/day)	0.0000631	0.005	0.0946	0.0000225	0.001	0.3532
Serum 25(OH)D (nmol/L)	0.000622	0.004	0.1322	0.000914	0.020	0.0005
Serum 1,25(OH)$_2$D (pmol/L)	−0.000405	0.015	0.0028	−0.000246	0.013	0.0046
Serum intact PTH[a] (pmol/L)	−0.0183	0.002	0.3298	−0.0357	0.015	0.0029
Serum osteocalcin (ng/ml)	−0.00868	0.054	<0.0001	−0.00532	0.050	<0.0001
Serum NTX (nmol BCE/L)	−0.113	0.043	<0.0001	−0.0655	0.035	<0.0001

Abbreviations: TUG, Timed "Up & Go"; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; PTH, parathyroid hormone; NTX, type I collagen cross-linked N-telopeptides.
[a] Logarithmically transformed.

QDR4500a (Hologic Inc., Bedford, MA, USA) by a single, trained X-ray technician. The in vivo coefficients of variation (CVs) of the BMD measurements were 0.3% for the lumbar spine and 0.6% for the femoral neck.

Physical examination

The grip strength of each hand was measured once with a digital hand dynamometer, and the average value of both hands was adopted. Isometric thigh muscle strength of both legs together was measured with a leg muscle dynamometer (T.K.K.5710g, Takei Scientific Instruments, Co., Ltd., Niigata, Japan). Walking ability (walking time) was assessed by the timed "Up & Go" (TUG) test [7]. Body height and weight of the subjects in light underwear were measured to the nearest 1 mm and 100 g, respectively. The body mass index (BMI) was calculated by dividing body weight (kg) by the square of body height (m^2).

Biochemical measurements

A 6-h-fasting blood specimen was drawn in the daytime. The specimen was immediately maintained at 4 °C. The serum was obtained within 1 day of collection by centrifugation at $1613 \times g$ for 10 min and stored at −80 °C until the biochemical analysis. The serum 25(OH)D concentration was determined by radioimmunoassay (DiaSorin, Stillwater, MN, USA) with an inter-assay CV value of 9.9%. The serum 1,25-dihydroxyvitamin D (1,25[OH]$_2$D) concentration was determined by radioimmunoassay (IDS Ltd., Boldon, England, UK), which has an inter-assay CV value of 12.8%. The serum intact PTH concentration was measured with a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Clemente, CA, USA), which has an inter-assay CV value of 1.5%. The serum osteocalcin (OC) concentration was determined by an immunoradiometric assay (Mitsubishi Kagaku Medical, Inc., Tokyo, Japan) with an inter-assay CV value of 6.6%. The serum type I collagen cross-linked N-telopeptides (NTX) concentration was determined by an enzyme-linked immunosorbent assay (Osteomark NTX Serum, Ostex International, Inc., Seattle, WA, USA), which had an inter-assay CV value of 2.8%.

Interview

Demographic, lifestyle, and nutritional information was obtained through interview. Age, reproductive history, medical history, and current medications were recorded. Current calcium intake was assessed with a previously validated food frequency questionnaire [8]. The correlation coefficient between values measured by this method and the conventional 3-day diet record was 0.668. Physical activity levels were assessed based on whether subjects engaged in the
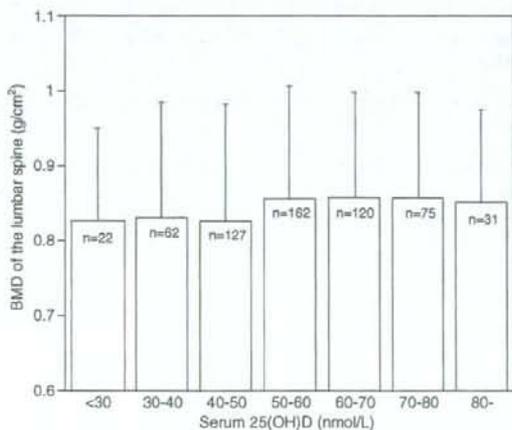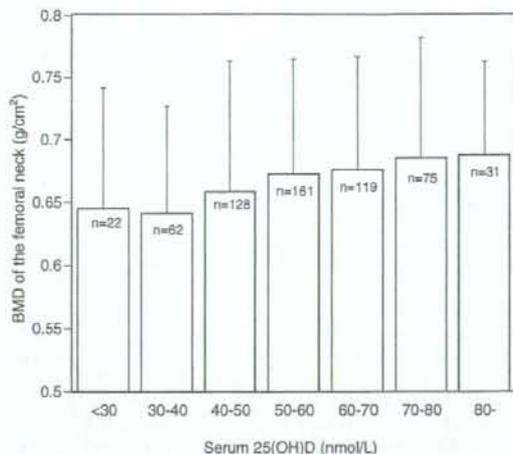

Fig. 2. Mean (plus SD) values of bone mineral density (BMD) of the femoral neck for each 10-nmol/L increment in the serum 25-hydroxyvitamin D (25[OH]D) concentration. BMD becomes higher as the 25(OH)D level becomes higher beginning from the 40- to 50-nmol/L group of serum 25(OH)D.

following three activities at least once a week: (1) housework, (2) light exercise, such as gate ball (or croquet), taking walks, and so on, as light activity, and (3) farmwork (or gardening), as moderate activity.

Statistical analysis

All continuous variables were checked for normality. TUG test, serum intact PTH, and NTX concentrations were skewed to higher values and were transformed logarithmically prior to conducting statistical tests. Categorical variables, such as "housework", "light exercise", and "farmwork" were coded as 0 for "no" and 1 for "yes". Student's t-test was used to test a difference in two mean values. Analysis of variance (ANOVA) was used to test differences among multiple mean values. ANOVA with Dunnett's multiple comparison was used to compare

Fig. 1. Mean (plus SD) values of bone mineral density (BMD) of the lumbar spine for each 10-nmol/L increment in the serum 25-hydroxyvitamin D (25[OH]D) concentration. The serum 25(OH)D concentration was not linearly associated with BMD at the lumbar spine ($P = 0.1322$), although 50 nmol/L may be an inflection point.
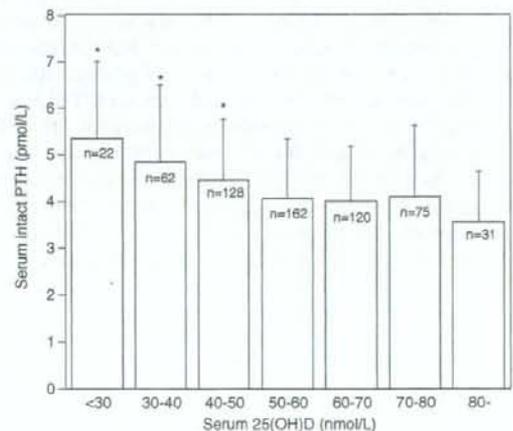

Fig. 3. Mean (plus SD) values of the serum intact parathyroid hormone (PTH) concentration for each 10 nmol/L increment in the serum 25-hydroxyvitamin D (25[OH]D) concentration. Mean serum intact PTH concentrations for 25(OH)D <30 nmol/L, 30–39 nmol/L, and 40–49 nmol/L, indicated with an asterisk (*), are significantly higher than those for serum 25(OH)D concentrations ≥50 nmol/L, as assessed by ANOVA with the Dunnett multiple comparison.

Table 3

Results of a stepwise multiple linear regression analysis predicting bone mineral density (BMD)

Independent variable	Regression coefficient (β)	Standard error	R^2	P value
BMD of the lumbar spine				
BMI (kg/m^2)	0.0115	0.0016	0.099	<0.0001
Age (years)	−0.00487	0.00117	0.058	<0.0001
Serum osteocalcin (ng/ml)	−0.00633	0.00152	0.041	<0.0001
Grip strength (kg)	0.00482	0.00146	0.016	0.0011
Calcium intake (mg/day)	0.0000904	0.0000344	0.012	0.0089
Years since menopause	−0.00142	0.00065	0.005	0.0279
Serum NTX[a] (nmol BCE/L)	−0.0434	0.0224	0.005	0.0535
BMD of the femoral neck				
BMI (kg/m^2)	0.00825	0.00010	0.112	<0.0001
Age (years)	−0.00521	0.00061	0.084	<0.0001
Serum osteocalcin (ng/ml)	−0.00422	0.00085	0.031	<0.0001
Serum 25(OH)D (nmol/L)	0.000705	0.000235	0.020	0.0029
Serum intact PTH[a] (pmol/L)	−0.0292	0.0107	0.013	0.0065
Grip strength (kg)	0.00215	0.00090	0.009	0.0167
Calcium intake (mg/day)	0.0000449	0.0000211	0.008	0.0336

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; NTX, type I collagen cross-linked N-telopeptides.

[a] Log-transformed values.

one mean value with other mean values. Simple linear regression analysis was used to identify predictors of BMD, indices of bone metabolism, including the log-transformed serum intact PTH, OC, and log-transformed NTX, and physical tests, including muscle strength and log-transformed TUG test as outcome variables. A stepwise multiple linear regression analysis was used to identify independent predictors of BMD. Candidate independent variables for the step-wise method were age, menopausal age, BMI, physical tests, lifestyle variables, calcium intake, the 25(OH)D, log-transformed serum intact PTH, OC, and log-transformed NTX concentrations. The serum 1,25(OH)$_2$D concentration was not included in the model because a negative association between serum 1,25(OH)$_2$D concentrations and BMDs was considered to be due to a compensatory increase of serum 1,25(OH)$_2$D concentrations for low bone mass [9,10]. Multiple logistic regression analyses were used to calculate adjusted odds ratios (ORs) of vitamin D insufficiency for "low BMD (t score ≤ −2.5 SD)". Test for linear trend was performed by using the logistic regression technique. Computations were performed by using the SAS statistical package (release 8.02, SAS Institute Inc., Cary, NC, USA). A P value less than 0.05 was considered statistically significant.

Results

The demographic and physical characteristics, nutritional intake, bone mass, and serum biochemical profiles are shown in Table 1. The proportion of subjects who had the serum 25(OH)D concentration less than 30 nmol/L and 50 nmol/L were 22/600 (3.7%) and 212/600 (35.3%), respectively. On the physical activity measure, 572 (95.3%) subjects did housework, 250 (41.7%) engaged in light activity, and 298 (49.7%) engaged in farmwork (moderate activity). "Low BMDs" (t score ≤ −2.5 SD) were observed in 106/599 (17.7%) of lumbar spines and 82/598 (13.7%) of femoral necks.

The results of the simple linear regression analyses with BMD as the outcome are shown in Table 2. The serum 25(OH)D concentration was not significantly associated with BMD of the lumbar spine but was positively associated with BMD of the femoral neck. Mean BMD at the lumbar spine for each 10-nmol/L increment in the serum 25(OH)D concentration is shown in Fig. 1. The serum 25(OH)D concentration was not linearly associated with BMD at the lumbar spine ($P=0.1322$). Mean BMDs at the femoral neck for each 10-nmol/L increment in the serum 25(OH)D concentration are shown in Fig. 2. BMD increases as the 25(OH)D concentration increases beginning from the 40- to 50-nmol/L group of serum 25(OH)D.

The serum 25(OH)D concentration was negatively associated with the log-transformed serum intact PTH concentration ($\beta = -0.00543$, $R^2=0.061$, $P<0.0001$). Mean serum intact PTH concentrations for each 10 nmol/L increment in the serum 25(OH)D concentration are shown in Fig. 3. Mean serum intact PTH concentrations for serum 25(OH)D <30 nmol/L, 30–39 nmol/L, and 40–49 nmol/L were significantly higher than a probable baseline intact PTH concentration, i.e., the mean intact PTH concentration for serum 25(OH)D concentrations ≥ 50 nmol/L. A linear association between calcium intake and the log-transformed serum intact PTH concentration was of borderline significance ($P=0.0611$). The log-transformed serum intact PTH concentration was significantly associated with both serum OC ($\beta=1.29$, $R^2=0.011$, $P=0.0102$) and log-transformed NTX ($\beta=0.0749$, $R^2=0.008$, $P=0.0302$) concentrations.

Table 4

Odds ratios (OR) and 95% confidence intervals (CI) for "low bone mineral density (BMD) (t score ≤ −2.5 SD)" according to levels of serum 25(OH)D

	Levels of serum 25(OH)D (nmol/L)						P for trend
	<30 ($n=22$)	30–40 ($n=62$)	40–50 ($n=127$)	50–60 ($n=162$)	60–70 ($n=120$)	≥70 ($n=106$)	
Lumbar spine							
Prevalence of low BMD (%)	18.2	22.6	25.2	16.1	15.0	11.3	
Unadjusted OR	1.61	1.99	2.23	1.42	1.33	1 (ref.)	0.0109
95% CI	0.57–4.52	0.99–4.03	1.21–4.10	0.75–2.69	0.67–2.62		
Adjusted[a] OR	3.03	2.44	3.02	1.32	1.48	1 (ref.)	0.0173
95% CI	0.57–16.02	0.84–7.12	1.31–6.97	0.59–2.99	0.61–3.59		
Femoral neck							
Prevalence of low BMD (%)	18.2	21.0	23.4	11.2	9.2	5.7	
Unadjusted OR	3.21	3.70	4.14	1.98	1.63	1 (ref.)	<0.0001
95% CI	0.99–10.44	1.48–9.25	1.79–9.57	0.81–4.81	0.63–4.26		
Adjusted[a] OR	2.86	3.59	7.55	2.07	1.40	1 (ref.)	0.0017
95% CI	0.28–29.03	1.06–12.11	2.45–23.24	0.74–5.80	0.45–4.35		

[a] Adjusted for age, menopausal age, BMI, calcium intake, grip strength, log-transformed intact PTH, OC, and log-transformed NTX.

The results of the stepwise multiple regression analysis are shown in Table 3. BMI was the predominant independent variable, followed by age and serum OC concentration for both BMDs of the lumbar spine and femoral neck. The serum 25(OH) D concentration was independently associated with BMD of the femoral neck, although its R^2 was smaller than those of BMI, age and serum OC concentration.

Table 4 shows ORs for "low BMD (t score ≤ -2.5 SD)" by level of serum 25(OH)D. After adjustment for model covariates, prevalence of low BMD for the lumbar spine was significantly higher in the 40- to 50-nmol/L group compared to the reference group (≥ 70 nmol/L). Similarly, a significantly higher prevalence of low BMD of the femoral neck was observed in the 30- to 40-nmol/L and 40- to 50-nmol/L groups compared to the reference group (≥ 70 nmol/L). The serum 25(OH)D concentration was not significantly associated with the serum OC concentration ($P=0.1715$) or the serum NTX concentration ($P=0.2355$). The lack of these associations remained after subjects were restricted to those with serum 25(OH)D concentrations <50 nmol/L ($P=0.4839$ for serum OC and $P=0.9574$ for serum NTX).

The serum 25(OH)D concentration is generally believed to be associated with physical strength. However, the serum 25 (OH)D concentration was significantly associated with neither thigh muscle strength ($P=0.1144$), grip strength ($P=0.3131$), nor the TUG test ($P=0.6140$). Even when comparing in these three physical variables between lower and higher subgroups by using any thresholds, there were no significant differences in any variables between them.

Discussion

This is the first large-scale epidemiologic study exploring a possible association between vitamin D status and bone mass, bone metabolism, or physical strength in postmenopausal Asian women. The mean serum 25(OH)D concentration (55.6 nmol/L) and prevalence of vitamin D insufficiency observed in this population were similar to those of other populations of ambulant Japanese elderly women [11,12]. The vitamin D status of ambulant elderly Japanese, including this study population, is well maintained even in winter, due in part to high dietary intake of vitamin D from fish [11,13]. This study demonstrated that the serum 25(OH)D concentration was linearly associated with BMD of the femoral neck in subjects with a serum 25(OH)D concentration of 30 nmol/L or higher. This finding is in accordance with the result of a large epidemiologic study recently conducted [4] and supports a rationale that the serum 25(OH)D levels should be maintained 75–80 nmol/L or higher [6,14]. By contrast, an association between the serum 25(OH)D concentration and BMD of the lumbar spine was not significant. This discrepancy has not been frequently reported in the literature, but may be explained by the fact that vitamin D status affects cortical bone more than spongy bone. This hypothesis is supported by Stone et al.'s [15] finding that lower 25(OH)D levels are associated with hip but not calcaneal bone loss. Regarding the association between the serum 25(OH)D concentration and BMD of the lumbar spine, 50 nmol/L appears

to be an inflection point (Fig. 1). This study may have failed to detect a true association due to the relatively small number of subjects at high 25(OH)D levels. Further studies should address this issue.

The present study showed that the serum 25(OH)D concentration of 50 nmol/L or lower was associated with low BMD (t score ≤ -2.5 SD) of both the lumbar spine and femoral neck (no significant increase in the prevalence of low BMD was observed in the <30 nmol/L group due to limited sample size). Study findings also suggest that vitamin D insufficiency is more strongly associated with low BMD in the femoral neck than in the lumbar spine.

Despite the significant associations observed between serum 25(OH)D concentration and BMD, the low R^2 values associated with vitamin D status in multivariate analysis indicate that it accounted for only a small proportion of the variance in BMD in the study population. Results of the present study are in line with the findings of two recent population-based investigations targeting postmenopausal women. The Rancho Bernardo Study [16] showed a slight but significant association between serum 25(OH)D and femoral BMD, and the OFELY Study [17] showed serum 25(OH)D not to be a significant determinant of bone loss. On the other hand, there have been two clinic-based studies in which the serum 25(OH)D concentration was correlated moderately with both spinal and femoral BMDs in postmenopausal women [18,19]. As such, the strength of the association between vitamin D status and BMD seems to depend on which population is targeted.

Numerous studies have shown an inverse association between the serum 25(OH)D and intact PTH serum concentrations [20–22]. The present study confirmed such an association with a threshold of 50 nmol/L of the serum 25(OH)D concentration for elevated serum intact PTH concentrations. This finding suggest that maintenance of serum 25(OH)D concentrations of at least 50 nmol/L is essential for maintaining bone health in postmenopausal Japanese women.

This study failed to confirm an association between serum 25 (OH)D concentration and markers of bone turnover. Gallagher et al. [23] also reported no or only a slight association between the serum 25(OH)D concentration and markers on bone turnover in a healthy elderly population. On the other hand, Jesudanson et al. [24] showed a negative association between serum 25(OH)D concentration and serum bone resorption markers and alkaline phosphatase levels in postmenopausal women attending an osteoporosis clinic. Furthermore, an inverse relationship between serum 25(OH)D and markers of bone turnover was found in postmenopausal women with established osteoporosis [25]. Taken together, these studies suggest an association between the serum 25(OH)D and markers of bone turnover may be observed in frail populations, such as osteoporotic women, but not in the general population of postmenopausal women.

Our study also demonstrated that serum intact PTH is associated with BMD of the femoral neck, but not with BMD of the lumbar spine. The lack of the association with the lumbar spine may be due to the fact that PTH affects cortical bone mass [26] to a greater extent than spongy bone mass or because PTH does not have as great of an effect on bone mass in elderly Asian

populations compared to their European counterparts [20]. Moreover, BMD of the femoral neck was independently associated with serum PTH and 25(OH)D, which suggests that each plays an independent role in bone metabolism and bone mass. PTH may affect BMD partly via increased bone turnover because high serum PTH was associated with both serum OC and NTX in this study. On the other hand, serum 25(OH)D may affect BMD not via increased bone turnover, as serum 25(OH)D did not link to bone turnover markers in this study but probably via increased calcium absorption in the intestine. The cross-sectional nature of this study has limitations in its ability to make causal relationships, and this hypothesis should be confirmed by a longitudinal study.

Low levels of vitamin D have been reported to be associated with impaired physical functions [27,28]. To the contrary, the present study failed to demonstrate such an association between vitamin D status and muscle strength or the TUG test. The lack of the associations in this study may be due to relatively good vitamin D status (mean serum 25[OH]D concentration, 55.6 nmol/L), the study population being relatively young (mean age, 64.5 years), or ethnicity [29].

The elderly Japanese population has some characteristics in terms of diet and bone health that make them different from other general populations. They have lower calcium intake and higher vitamin D intake than elderly whites [12]. In the present population, 95% of the subjects had total calcium intake of less than 800 mg/day, a daily calcium requirement in Japan [30]. Their low calcium intake (527 mg/day) might diminish an effect of vitamin D on bone, and increase of calcium intake is hypothesized to alter strength of the association between vitamin D status and bone mass.

This study had some limitations. This study employed a cross-sectional design, which is limited in its ability to detect causal relationships. An intervention trial is needed in order to establish causality. In addition, subjects' participation rate of this study was approximately 50%, and thus selection bias may have occurred. For example, it is likely that healthier or more active women tended to participate in this study. Generalizations of our results to other populations should thus be made with caution.

In summary, the present study was the largest study to date to examine the relationship between vitamin D levels and bone health among Asian postmenopausal women. Our results suggest 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. While significant associations were observed between vitamin D status and BMD of the femoral neck, the contribution of vitamin D status to BMD is relatively small, suggesting a role for other factors in low bone mass.

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ORIGINAL ARTICLE

High prevalence of vitamin K and D deficiency and decreased BMD in inflammatory bowel disease

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Abstract

Summary Vitamin K and D deficiency and decreased bone mineral density (BMD) were highly prevalent in patients with inflammatory bowel disease (IBD), especially Crohn's disease (CD). Dietary intakes of these vitamins, however, were above the Japanese adequate intakes in IBD patients, suggesting that malabsorption is the basis for hypovitaminosis K and D and decreased BMD.

Introduction We have studied the possible involvement of vitamin K and D deficiency in the pathogenesis of decreased BMD in IBD.

Methods Seventy patients with IBD were evaluated for their BMD; plasma levels of vitamin K; phylloquinone (PK), menaquinone-7 (MK-7), and 25OH-D; serum PTH, protein induced by vitamin K absence (PIVKA-II), and undercarboxylated osteocalcin (ucOC) levels; and their food intake.

Results Compared with ulcerative colitis (UC) patients, CD patients had significantly lower plasma vitamin K and 25OH-D concentrations; significantly higher serum levels of PTH, PIVKA-II, and ucOC; and significantly lower BMD scores at almost all measurement sites. More IBD patients were vitamin K deficient in bone than in liver. Multiple regression analyses revealed that low plasma concentrations of vitamin K and 25OH-D were independent risk factors for low BMD and that they were associated with the patients' fat intake, but not with their intake of these vitamins.

Conclusion IBD patients have high prevalence of decreased BMD and vitamin K and D deficiency probably caused by malabsorption of these vitamins.

Keywords Inflammatory bowel disease · Malabsorption · Vitamin K · Vitamin D

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC), collectively termed inflammatory bowel disease (IBD), are often associated with osteoporosis, the pathogenesis of which is considered to be multifactorial including inflammatory disease process, low body weight, calcium and vitamin D deficiency, and glucocorticoid use [1–5]. In this paper, we focused our attention to the possible involvement of vitamin K and D deficiency in IBD-induced osteoporosis based on the following considerations.

Vitamin K has received far less attention than vitamin D in the development of IBD-related osteoporosis [6]. The most fundamental role of vitamin K is to work as the coenzyme of hepatic γ-carboxylation of four of the blood coagulation factors [7]. Recent evidences suggest that

vitamin K is also essential in the extrahepatic tissues including skeleton and vasculature [8]. Fracture risk was increased in subjects with low vitamin K intake [9, 10] or increased serum undercarboxylated osteocalcin (ucOC) level, which is a sensitive marker for skeletal vitamin K deficiency [11, 12]. Furthermore, recent metaanalysis has shown that vitamin K treatment decreased fracture incidence [13]. These findings prompted us to study both vitamin K and D status in IBD patients.

Next, the vitamin K and D status of IBD patients has been studied by evaluating their food intake [14, 15] or by measuring circulating level of these vitamins [6, 16–18], but rarely by both [19, 20]. Patients with IBD have been reported to be at high risk of malabsorption of these vitamins due to intestinal inflammation or intestinal resection in some patients [6, 18, 21–24]. Therefore, the patients' intake of these vitamins may be discrepant from their circulating levels. Thus, we considered it mandatory that the vitamin K and D status of IBD patients should be evaluated by studying both the patients' intake and plasma levels.

In this paper, we have studied bone mineral density (BMD) at various sites, measured plasma concentrations of vitamin K and D as well as markers for their deficiency, and evaluated the patients' food intake to clarify the possible involvement of vitamin K and D deficiency in IBD-induced bone loss.

Materials and methods

Subjects

Seventy outpatients with IBD (CD, 29 and UC, 41) attending the Gastroenterology Clinic at Kyoto University Hospital participated in the study. Excluded from the study were patients already treated for osteoporosis with drugs such as bisphosphonates, calcium, vitamin K, or vitamin D. None had history of fragility fractures. Consent to participate in this study was obtained after explanation of the objective and protocol of this study. All subjects except two with CD and one with UC were receiving 5-aminosalicylic acid. Eight patients with CD and 17 with UC were under oral glucocorticoid therapy. Immunosuppressive drug was prescribed to 19 patients with CD and eight patients with UC. Three patients with CD, but none with UC, were on combined therapy of infliximab, oral glucocorticoid, and immunosuppressive drug. None of them were under warfarin therapy.

Measurement

Biochemical measurements

Plasma samples were stored at −30°C with protection from light until analyzed. Plasma vitamin K_1 (phylloquinone [PK]) and K_2 (menaquinone-7 [MK-7]) levels were determined by high-performance liquid chromatography–tandem mass–mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS) using a HPLC system (Shimadzu, Kyoto, Japan) and API3000 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) with ^{18}O-labeled vitamin K as the internal standard [25]. Plasma concentration of 25OH-D was measured by radioimmunoassay (RIA) (DiaSorin, Stillwater, MN, USA). This study was done between September and November to minimize the seasonal variation in serum 25OH-D levels. Serum intact PTH was measured by a fully automated immunochemilumetric assay (Nichols Institute Diagnostics, San Clemente, CA, USA) with 15–55 pg/mL as the reference range in Kyoto University Hospital. Serum protein induced by vitamin K absence (PIVKA-II) and ucOC levels were measured by electrochemiluminescent immunoassay (ECLIA; Sanko Junyaku, Tokyo, Japan) as the markers of hepatic and skeletal vitamin K deficiency, respectively. Serum NTX-I and bone specific alkaline phosphatase (BAP) levels were measured by enzyme immunoassay (EIA) (Mitsubishi Chemical Medience, Tokyo, Japan)

BMD measurement

BMD was measured at the lumbar spine (L1–4), femoral neck, total hip, and distal one-third of nondominant radius with dual-energy X-ray absorptiometry (QDR-2000, Hologic, Waltham, MA, USA). BMD (g/cm^2) values thus obtained were expressed as T or Z score. The diagnosis for osteoporosis was made according to the World Health Organization criteria with T score below −2.5 SD and between −2.5 and −1.0 SD being diagnostic of osteoporosis and osteopenia, respectively [26].

Dietary intake

Dietary information was obtained from 1-day dietary record completed by the patients [27]. Based on these records, their intake of energy and nutrients was calculated using a software (Healthy Maker Pro 501, Mushroom Software, Okayama, Japan).

Statistical analyses

Statistical analyses were performed using the SPSS 15.0 J for Windows (SPSS Japan, Tokyo, Japan). The difference between two independent groups was analyzed by unpaired t test or Mann–Whitney test depending on normality. Multiple regression analyses were performed to determine independent risk factors for plasma vitamin K, 25OH-D levels, or BMD.