査回数に交互作用がみられた。結論として、高齢者イメージは児童の成長とともに一般には低下する可能性があるが、“REPRINTS”ボランティアとの交流頻度が高い児童では、1年後も肯定的なイメージを維持しうることが示唆された。

更に、A小学校の保護者への波及効果を検証した[11]。その結果、低および中学年保護者の回答の2年間の経時変化において、学校行事への協力についての「保護者の物理的負担の軽減」は、初回調査では低学年保護者の評価が中学年保護者に比べて有意に高く、かつ両群とも経時的に評価は向上した。「保護者の心理的負担の軽減」および「認知度」は、初回調査で両群に有意差は無く、ともに経時的に評価は向上した。つまり、2年間のボランティア活動により,“REPRINTS”ボランティアの活動の一部への評価は児童の学年を問わず高まった。

以上より、“REPRINTS”プログラムによる、高齢者ボランティアと児童の互恵的効果が検証されたのみならず、児童を媒介として、高齢者と保護者世代にまたがる三世代の信頼感が構築される可能性が示唆された。

ソーシャルキャピタルは地域における信頼、互恵的な規範、ネットワークから構成される概念である。保護者が高齢者ボランティアに感謝の念を抱き、その思いは、親の介護を意識する世代としては、高齢者福祉への理解につながるかもしれない。一方では、子育てが一段落した後には、ボランティアとして、地域や他の子どもに貢献しようとする人も現れる可能性がある。こうして、保護者世代の高齢者理解と自身のボランティアへのきっかけが生まれ、さらに子どもはそうした親の姿から多くのことを学ぶであろう。果たして、互恵的な交流は世代間で継承され、地域を支える人的資源として好循環し、ソーシャルキャピタルが醸成されることが期待される。

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Vitamin K_1 (Phylloquinone) or Vitamin K_2 (Menaquinone-4) Induces Intestinal Alkaline Phosphatase Gene Expression

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Vitamin K_1 (Phylloquinone) or Vitamin K_2 (Menaquinone-4) Induces Intestinal Alkaline Phosphatase Gene Expression

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Summary Alkaline phosphatase (ALP) hydrolyzes a variety of monophosphate esters into inorganic acid and alcohol at a high optimum pH (pH 8–10). Previously, we identified a significant increase of intestinal ALP (IAP) activity in the rat intestine on long-term dietary vitamin K supplementation. However, it was unclear whether the induction of ALP gene expression was caused by vitamin K intake. In the present study, we examined the effects of vitamin K on IAP gene expression. A total of 21 male ICR strain mice (7 wk old) were divided into three groups: control, PK, and MK groups. Mice were orally administered a 0.1-mL solution of physiological saline in the control group, phylloquinone (3 mg/kg mouse) in the PK group, and menaquinone-4 (3 mg/kg mouse) in the MK group. Four hours after administration, we determined the ALP activity of the intestinal mucosa in three areas (duodenum, jejunum, and ileum). In the MK groups, the levels of ALP activity in the jejunum increased significantly compared with the control. Moreover, reverse transcription-polymerase chain reaction (RT-PCR) analysis using specific primers revealed that IAP mRNA expression was significantly enhanced in the jejunum in both PK and MK groups. Interestingly, vitamin K administration also increased the expression of pregnane X receptor mRNA. This is the first report concerning IAP mRNA expression induced by oral administration of vitamin K. The results support the possible involvement of vitamin K in the regulation of IAP mRNA expression as a novel pharmacological effect of vitamin K.

Key Words alkaline phosphatase, phylloquinone, menaquinone, mice, intestine

Alkaline phosphatase (ALP, EC 3.1.3.1) is an enzyme containing zinc which hydrolyzes monophosphate esters into inorganic phosphoric acid and alcohol at a high optimal pH (pH 8–10). The enzyme is distributed widely throughout the living world from bacteria to animals, excluding plants, and it exists in various tissues such as the intestine, liver, kidney, bone, placenta, stomach, and leukocytes.

In humans, four kinds of ALP isozyme have been identified: tissue-nonspecific ALP (liver/bone/kidney: TNSALP), intestinal ALP (IAP), placental ALP, and germ cell ALP (*1–4*). The TNSALP gene is located on chromosome 1 and consists of 12 exons and 11 introns, with the coding sequence beginning in the second exon.

A single gene for human IAP has been isolated, and the multiple forms of mRNA encoding human IAP are due to differences in polyadenylation (*2*). Although most species express a single IAP, several kinds of IAP have been identified in three species: the mouse (*5*), rat (*6, 7*), and cow (*8*).

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In rats, ALP is classified into two types: TNSALP and IAP. IAP is present in the membrane surrounding neutral fat droplets in the microvilli of the intestinal mucosa during fat absorption, and is thought to transport dietary lipids from the intestinal tract into the circulation as a component of unilamellar membranes called surfactant-like particles (SLPs) (*9*). Two different cDNA clones, IAP-I and IAP-II, for rat IAP were isolated by Lowe et al. (*6*) and Strom et al. (*7*), respectively. Strom et al. found that the expression of IAP-II mRNA was specifically enhanced by $1\alpha,25(OH)_2D_3$ administration. The two isozymes are products of two distinct genes and their cDNA sequences show 79% homology at the amino acid level. Functional differences between IAP-I and IAP-II were suggested by the differing regulation of the expression of the two mRNAs (*10*), as well as by structural and catalytic differences (*11*).

In mice, five different ALP loci have been identified: TNSALP, IAP, embryonic ALP (EAP), *Akp6*, and *Akp-ps1*. These ALP genes code for different proteins: *Akp2* encodes TNSALP, *Akp3* encodes IAP, *Akp5* encodes EAP, *Akp6* encodes a novel IAP-like isozyme expressed globally in the gut (thus called gIAP), and *Akp-ps1* encodes

the inactive pseudo-type ALP (*5*, *7*).

Previously, we reported the enhanced effects of vitamin K on IAP activity in rats (*12*). Sprague-Dawley rats (6 wk old) were divided into three groups: a control (AIN-93M diet), phylloquinone (PK: 600 mg/kg diet), and menaquinone-4 (MK-4: 600 mg/kg diet) diet group. After 3 mo of feeding of vitamin K, the animals were fasted overnight. The small intestine was removed and divided into five segments. In each segment, both PK and MK-4 increased IAP activity (*12*).

Vitamin K acts as a cofactor for γ-glutamyl carboxylase (GGCX), and is well-known to participate in the activation of blood coagulation factors and bone mineralization (*13*). All forms of vitamin K have 1,4-naphthoquinone as a common ring structure, and natural vitamin K exists in two molecular forms, vitamin K_1 (phylloquinone: PK) and vitamin K_2 (menaquinone: MK-*n*). PK is abundant in green vegetables in a compound with a phytyl side chain. Vitamin K_2 is classified into MK-1–14 due to the repeat structure of the side chain, with isopren comprising the side chain. MK-4 shows marked physiological activities as a vitamin K, and is included in many animal-based foods such as meat. Recent studies have demonstrated the possibility that vitamin K regulates the expression of bone-related genes such as ALP through steroid X receptor (SXR, also termed pregnane X receptor: PXR) (*14*).

In the present study, we examined whether the enhancing effect of PK or MK-4 administration on IAP activity occurs via the intestinal mucosa directly, and we revealed the effects of the oral administration of PK or MK-4 on the expression of IAPs (*Akp3* and *Akp6*) and PXR in the mouse intestine.

MATERIALS AND METHODS

Experimental animals. The care and use of mice in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals, and the study protocol was approved by the Institutional Review Board of Japan Women's University. A total of 21 male ICR strain mice (7 wk old) were used (31.1±0.2 g). They were fasted overnight with free access to water. On the following day, the animals were given 0.1 mL of solution via an intragastric tube: vehicle (physiological saline) for the control group (Cont.), PK (3 mg/kg mouse) for the PK group, and MK-4 (3 mg/kg mouse) for the MK group. The molecular weights of PK ($C_{31}H_{46}O_2$: MW=450.7) and MK-4 ($C_{31}H_{40}O_2$: MW=444.7) are very similar. PK and MK-4 were kindly supplied by Eisai Co., Ltd. (Tokyo, Japan).

Serum and tissue sampling. Four hours after administration, blood was collected from the abdominal aorta under ether anesthesia, and perfusion with saline was performed until the liver was blanched, in order to minimize the blood contamination of tissue samples. The small intestine was removed and divided into three regions. From the pylorus, we took the first 1 cm as the duodenum, and then separated the remaining part into the jejunum and ileum. The segments were slit longitudinally, rinsed with ice-cold saline, and scraped from the mice just after dissection. Each sample was homogenized using a Polytron homogenizer (Kinematica, Switzerland) with 10 mM Tris-buffered saline containing 1% Triton X-100 (pH 7.3) and 1 mM phenylmethylsulfonylfluoride (PMSF). The supernatant obtained after centrifugation at 7,000 ×*g* for 15 min was used as the enzyme extract.

Enzyme assay. ALP activity was determined with 10 mM *p*-nitro-phenylphosphate as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediole HCl buffer containg 5 mM $MgCl_2$, pH 10.0, at 37°C, as previously reported (*15*). To analyze the biochemical properties of ALP, an inhibitory assay using levamisole (Lev) and L-phenylalanine (L-Phe) and a thermostability assay were performed, as previously described (*15*).

The enzyme activity was defined as the rate of hydrolysis of *p*-nitro-phenylphosphate and expressed in units (U=μmol *p*-nitro-phenol formed/min).

Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyacrylamide gel (7.5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to the method of Weber et al. (*16*). After electrophoresis, ALP isozymes separated in the gel were stained by the coupling of β-naphthyl-phosphoric acid monosodium salt with Fast Violet B salt (*17*).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the intestinal mucosa was extracted employing the acid guanidinium thiocyanate-phenol-chloroform method (*18*). As a template for PCR, single-strand cDNA was prepared from 1 μg of total RNA using Ready-to-go You-Prime First-Strand Beads (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). PCR primers were used for *Akp3* (*19*), *Akp2* (*20*), *Akp5* (*21*), and PXR (*22*). In order to detect *Akp6*, sense (*Akp6*-up) and anti-sense (*Akp6*-down) primers were designed on the basis of the *Akp6* nucleotide sequence (GenBank: NCBI sequence data: AK008000). *Akp6*-up spans nucleotide positions 882–902 and *Akp6*-down spans 1,333–1,354 (*23*). The PCR conditions were as follows: 5 cycles at 94°C (1 min), 50°C (1 min), and 72°C (1 min), and 25 cycles at 94°C (30 s), 55°C (30 s), and 72°C (30 s), followed by 10 min at 72°C. Negative controls were performed with each RT-PCR reaction, omitting the template. The efficiency of reverse transcription was verified by the detection of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse:5′-TCC ACC ACC CTG TTG CTG TA-3′), as previously described (*23*).

The amplified samples were analyzed using 5.25% polyacrylamide gel electrophoresis (PAGE). The gels were stained with ethidium bromide and observed under UV light. The band intensity on PCR photographs was quantified by densitometry (AE6920M, ATTO, Tokyo, Japan). The PCR product was normalized to the intensity of the band for the house-keeping gene GAPDH, and is expressed as a ratio of the relative band intensity.

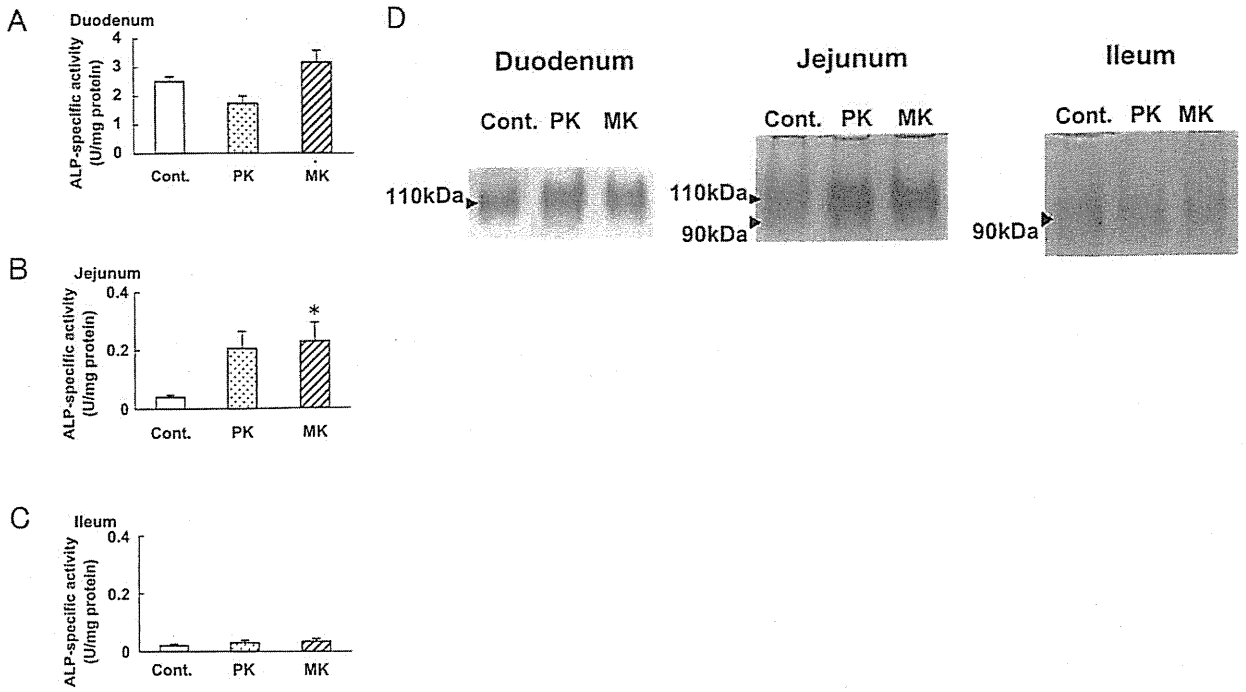

Fig. 1. ALP-specific activities of the duodenum (A), jejunum (B), and ileum (C). Results are the mean±SE of 7 animals. Significant difference between the MK and control groups (*$p<0.05$). D: Mouse intestinal ALP isozymes separated by polyacrylamide gel electrophoresis. The gels was stained for ALP activity with a β-naphtyl-phosphoric acid monosodium salt, Fast violet B salt. Cont.: control, PK: phylloquione, MK: menaquinone-4.

Statistical analyses. Values are shown as the mean± standard error (SE).

Dunnet's multiple comparison test was used after ANOVA to compare the significance of differences among the control and PK or MK. Differences were considered significant at $p<0.05$. Analysis was conducted using SPSS 18.0J (SPSS, Inc., Chicago, IL, USA).

RESULTS

ALP activity

To examine whether IAP was secreted from the intestinal mucosa on the oral administration of PK or MK, we measured the levels of serum ALP activity (mU/mL) in the control, PK, and MK groups, being 13.7±1.2, 10.3±2.0, and 14.1±1.8 (mean±SE), respectively, showing no significant differences among these groups.

ALP-specific activities in the intestine are shown in Fig. 1. There were no significant differences in ALP activities among these groups in the duodenum (Fig. 1A) and ileum (Fig. 1C). As presented in Fig. 1B, ALP activity of the MK group in the jejunum was significantly higher compared with the control group ($p<0.05$).

Molecular weight determination by SDS-PAGE

The molecular weights of ALPs of each intestinal segment were estimated employing SDS-PAGE analysis. As shown in Fig. 1D, the 110-kDa band of the major ALP isozyme was detected in the duodenum among these groups. In the jejunum, ALP enzymes were separated into two bands of 110 and 90 kDa, and the intensity of their enzymatic activity increased markedly in both PK and MK groups, similarly to the results regarding the specific ALP activity in the jejunum. In the distal part of the intestine (ileum), the ALP isozyme showed a main band of 90 kDa among these groups. No additional band was observed in any intestinal samples among the groups.

Table 1. Inhibitory effects of levamisole, L-phenylalanine and heat inactivation of ALP preparations of the jejunum.

Groups	Relative activity(%)		
	Levamisole (1 mM)	L-Phenylalanine (20 mM)	Heat inactivation (60°C 10 min)
Cont.	95.2±1.6	23.0±1.0	42.4±3.4
PK	94.2±2.1	23.5±1.4	46.1±2.3
MK	95.5±1.6	24.2±2.0	45.9±2.5

Each value represents mean±SE ($n=7$).
The ALP activity was assayed based on the rate of *p*-NPP hydrolysis. The effect of the inhibitor was determined in the presence of 5 mM $MgCl_2$ in the assay mixture. Remaining ALP activity with inhibitors or after heat treatment is expressed as a percent of non-treated controls. Results are the mean±SE of 7 animals.

Properties of ALP in the intestine

The enzymatic properties of ALP preparations of the jejunum which increased significantly on PK or MK administration were investigated employing an inhibition experiment with levamisole (Lev) and L-phenylalanine (L-Phe) and through a thermo-stability test. It is well known that IAP activity is not inhibited by Lev and is more stable to L-Phe and more heat-stable than TNSALP. As shown in Table 1, there was no significant

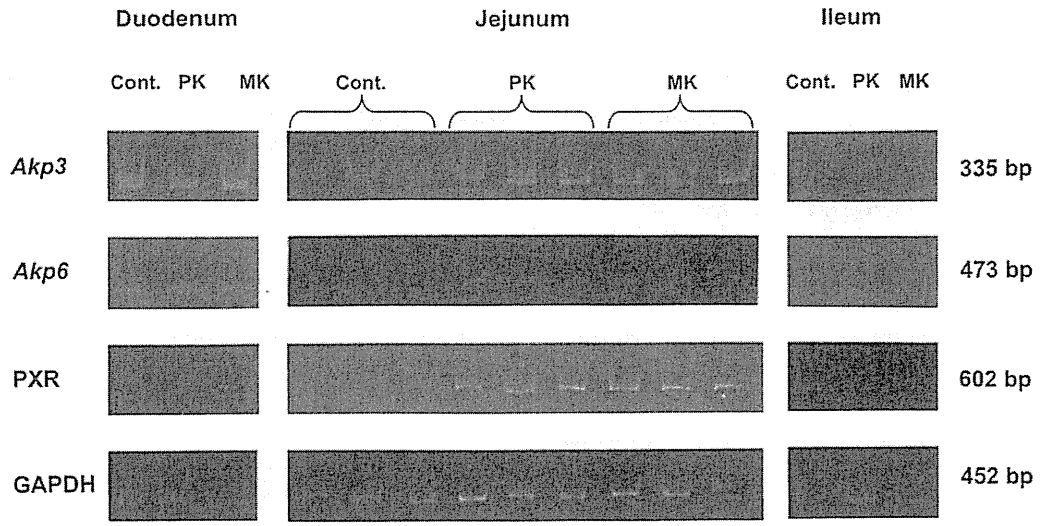

Fig. 2. Detection by RT-PCR of mRNAs for *Akp3*, *Akp6*, PXR, and GAPDH in the duodenum, jejunum, and ileum. PCR products were electrophoresed in a 5.25% polyacrylamide gel. Cont.: control, PK: phylloquinone, MK: menaquinone-4.

difference in the relative activities among these groups. We confirmed that these jejunum ALP preparations were effectively inhibited by L-Phe but not by Lev, and were heat-stable (60°C, 10 min), corresponding to the property of other mammalian intestinal type ALPs.

RT-PCR analysis of ALP mRNA expression

RT-PCR-based detections of mRNA for *Akp3*, *Akp6*, and PXR in the duodenum, jejunum, and ileum are shown in Fig. 2. The PCR products of *Akp3* (335 bp) were detected in the duodenum and jejunum. The PCR products of *Akp6* (473 bp) were detected in the duodenum, jejunum, and ileum. The PCR products of PXR (602 bp) were detected in the duodenum, jejunum, and ileum. The intensities of mRNA expression of *Akp3* and *Akp6* were very similar both in the duodenum and ileum among the three groups (Cont., PK, and MK groups). The intensities of mRNA expression of *Akp3*, *Akp6*, and PXR were enhanced in both the PK and MK groups compared with the control group in the jejunum.

PCR products of *Akp2* (198 bp) and *Akp5* (500 bp) were not detected in any of these intestinal samples.

In order to compare these intensities of mRNA expression in the jejunum, we determined the relative density of the PCR products of the mouse jejunum. As shown in Fig. 3A, the intensities of *Akp3* expression increased significantly in the PK group compared with the control group ($p<0.01$). Furthermore, the intensities of *Akp6* expression were also increased in both the PK and MK groups compared with the control group ($p<0.01$, $p<0.05$, respectively) (Fig. 3B). Interestingly, the intensities of PXR expression in both the PK and MK groups were significantly higher than in the control group ($p<0.05$, $p<0.01$, respectively) (Fig. 3C).

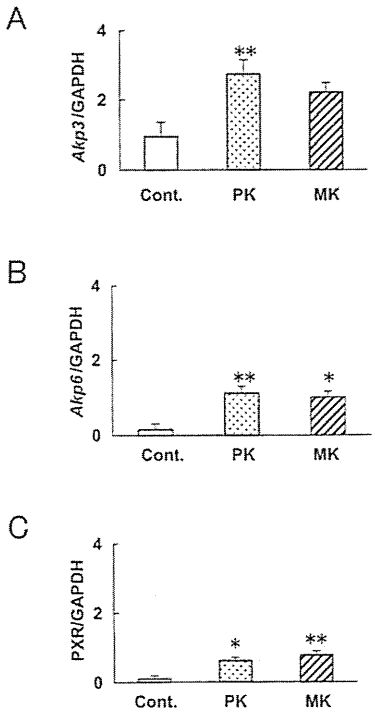

Fig. 3. The relative density of PCR products in the mouse jejunum. The diagrams show the relative density of the PCR products. A: The relative density of PCR products for *Akp3*. B: The relative density of PCR products for *Akp6*. C: The relative density of PCR products for PXR. Results are the mean±SE of 4 animals. Significant difference between the PK or MK and control groups ($^*p<0.05$, $^{**}p<0.01$). Cont.: control, PK: phylloquinone, MK: menaquinone-4.

DISCUSSION

Previously, we reported that several dietary factors such as fat-feeding, vitamin K, and lactose increased IAP activities in rats (*12*, *15*, *24*). The high-level activity of IAP, which localizes at the brush border of intestinal epithelium cells, suggests the participation of this enzyme in the transport of nutrients.

Recently, we reported that both long-term dietary PK and MK-4 supplementations enhance IAP activity in rats (*12*). After 3 mo of feeding, we measured IAP activity by dividing it into five segments. In each segment, both PK and MK-4 increased IAP, and the level of IAP activity in the proximal jejunum was significantly

higher than that in the control group ($p<0.05$) (*12*). To examine whether the enhancing effect of PK or MK-4 on IAP activity occurs via the intestinal mucosa directly, we performed an oral administration of PK or MK-4 using mice. In the present study, we discovered that the levels of mouse jejunum ALP activity were also significantly increased by the oral administration of PK or MK compared with the control group (Fig. 1B). In addition, we confirmed that the increased ALP isozymes induced by the oral administration of PK or MK showed similar biochemical properties to the typical intestinal type ALP, with no significant differences among these groups (Table 1).

By SDS-PAGE analysis, we detected a 110-kDa ALP enzyme in the duodenum and 90-kDa ALP enzyme in the ileum (Fig. 1D). Both the 110- and 90-kDa ALP enzymes were detected in the jejunum, and the enzymatic activities of these bands were enhanced by the oral administration of PK or MK. The product of the *Akp3* gene was expressed specifically in the duodenum, and the product of *Akp6* was expressed through the small intestine (*5*). Therefore, we considered that the 110- and 90-kDa ALP enzymes may correspond to the IAPs encoding *Akp3* and *Akp6*, respectively.

We then performed RT-PCR analysis in order to examine the expression of IAPs (*Akp3* and *Akp6*) in the mouse jejunum. PCR products for *Akp3* and *Akp6* mRNAs in the jejunum were detected, and a significant increase in the PCR products of *Akp3* due to the oral administration of PK was observed (Fig. 3A). Moreover, a significant increase in the PCR products of *Akp6* due to the oral administration of PK or MK-4 was also observed (Fig. 3B). These results suggest that the induction of *Akp3* and *Akp6* may be regulated by PK or MK-4.

As the results of RT-PCR, the expression of mRNA for PXR was detected in the duodenum, jejunum and ileum, and it was enhanced significantly in both the PK and MK groups in the jejunum compared with the control group (Fig. 3C). Interestingly, the intestinal segment where the expression of mRNA for PXR by vitamin K had been enhanced corresponded to a similar segment where the expression of mRNA for IAP was enhanced.

Recent studies have revealed that vitamin K functions as a ligand for nuclear steroid and xenobiotic receptor (SXR), as well as a cofactor for γ-carboxylase (*25*). SXR is expressed predominantly in the liver and intestine, and it regulates transcription such as of cytochrome P450 (CYP) 3A4, which is an enzyme involved in drug metabolism, and MDR1 (multidrug resistance protein 1) which is activated by a diverse array of pharmaceutical agents including taxol, rifampisin, and clotrimazole (*26*, *27*). Ichikawa et al. identified novel SXR target bone-related genes that were regulated by MK-4 in osteoblastic cells using microarray analysis (*28*). Among extracellular matrix-related genes, they demonstrated that a small leucin-rich repeat proteoglycan, tsukushi, contributes to collagen accumulation (*28*).

We demonstrated for the first time that the oral administration of vitamin K (both PK and MK-4) enhanced the level of IAP mRNA expression in the mouse intestine, and PXR mRNA expression also increased. Further studies on the physiological functions of ALP and transcriptional regulation of ALP induction will provide useful data on the novel effect of vitamin K.

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Effects of long-term vitamin K_1 (phylloquinone) or vitamin K_2 (menaquinone-4) supplementation on body composition and serum parameters in rats

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ABSTRACT

Vitamin K is a cofactor for γ-glutamyl carboxylase, which is an essential enzyme for the γ-carboxylation of vitamin K-dependent proteins such as osteocalcin and matrix Gla protein. Although it has been suggested that vitamin K plays an important role in the improvement of bone metabolism, the relationship between dietary vitamin K intake and bone metabolism has not been thoroughly investigated. Moreover, vitamin K is thought to have other actions beyond influencing the γ-carboxylation status. In the present study, we examined the effects of the long-term addition of phylloquinone (PK) or menaquinone-4 (MK-4) to a control diet on bone mineral density, bone strength, body composition, and serum parameters in rats. A total of 23 female Sprague–Dawley strain rats (6 weeks old) were divided into three groups: basic control diet group, PK diet (PK: 600 mg/kg diet) group, and MK diet (MK-4: 600 mg/kg diet) group. Three months after starting the experimental diet, the addition of PK to the basic control diet significantly increased the bone mineral density (BMD) of the femur ($p<0.05$). In the MK group, there was no significant difference in the BMD of the femur. However, two types of bone strength parameter: the minimum cross-sectional moment of inertia and the polar moment of inertia, were significantly higher in the MK group than in the control ($p<0.05$, respectively). Furthermore, the femoral bone parameters (the width, dry weight and ash weight, and cortical, cancellous, trabecular, and total bone mineral contents) in the MK group were increased significantly compared with the control. Interestingly, the addition of PK or MK-4 significantly decreased the total fat accumulation ($p<0.01$ and $p<0.05$, respectively), and serum triglycerides were reduced by 48% in the PK group and 29% in the MK group compared with the control. There were no significant differences in the levels of serum calcium, phosphorus, alkaline phosphatase, growth hormone, insulin-like growth hormone-1, insulin-like growth hormone binding protein-3, and cross-linked N-teleopeptide of type I collagen among the three groups. This is the first study to demonstrate the effect of the long-term addition of PK or MK-4 to the control diet on body composition and serum parameters in an *in vivo* system using rats. Further studies on the mechanism of vitamin K supplementation in the regulation of bone metabolism would provide valuable data on the prevention of lifestyle-related disorders, including osteoporosis.

Introduction

Vitamin K was originally recognized as a factor involved in blood clotting, and is known to be important in bone metabolism. In nature, vitamin K exists in two forms: vitamin K_1 (phylloquinone: PK), and vitamin K_2 (menaquinone: MK-n), which comprises a family of naphthoquinones with differing numbers of isoprenoid residues (1 to 14) at the 3-position of naphthoquinone. PK is found in leafy, green vegetables, and menaquinone-4 (MK-4) is present in meat, eggs, and dairy products. Japanese fermented beans (referred to as natto) *Bacillus natto*, contain large amounts of menaquinone-7 (MK-7) synthesized by the bacteria.

Several epidemiologic studies have shown the association between biological makers of bone metabolism and vitamin K intake. A low dietary phylloquinone intake was associated with an increased risk of hip fracture in the elderly [1]. Kaneki et al. reported a significant inverse correlation between the incidence of hip fracture in women and the consumption of natto, one of the major sources of vitamin K_2 [2].

Vitamin K is a cofactor for vitamin K-dependent carboxylase, known as γ-glutamyl carboxylase (GGCX), which facilitates the post-translational modification of glutamic acid (Glu) to γ-carboxyglutamic acid (Gla) residues in selected proteins [3–7]. Three vitamin K-dependent proteins, osteocalcin (OC), matrix Gla protein (MGP), and protein S, are found in

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bone; OC is the most abundant [8–10]. OC is produced in osteoblasts, and fully carboxylated OC binds the calcium ions of hydroxyapatite [11].

Recently, Hosoi et al. clarified a significantly higher association between the single nucleotide polymorphism (SNP) of GGCX (R325Q, 974G>A) (rs699664) and bone mineral density (BMD) among postmenopausal women [12]. Moreover, we suggested that the requirement of vitamin K for γ-carboxylation may be different depending on the GGCX genotypes in healthy young males [13].

Vitamin K is thought to have other actions beyond the function of a coagulation factor associated with the γ-carboxylation status.

In this study, we investigated the effect of long-term PK or MK-4 supplementation on the BMD, bone strength, fat accumulation, serum parameters and bone metabolism markers in an *in vivo* system using rats.

Materials and methods

Experimental animals

The care and use of rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals. Six-week-old female Sprague Dawley rats were used, and all rats were initially fed the control diet (AIN-93 diet) [14] for eight days. Then, they were divided into three groups, and were each fed experimental diets for eighty-five days: control (Cont.) diet, vitamin K_1 (phylloquinone: PK) diet, or vitamin K_2 (menaquinone-4: MK-4) diet. The vitamin K diets were modified from AIN-93 and contained PK or MK-4 at 600 mg/kg, respectively [15]. PK and MK-4 were kindly supplied by Eisai Co., Ltd. (Tokyo, Japan). Ca, P, protein, and lipid contents were identical in these diets. The animals were housed individually in wire cages with free access to ion-exchanged distilled water. Twelve-hour light/dark cycles, a constant temperature (23 ± 1 °C), and constant humidity (50 ± 5%) were maintained. All rats were observed each day. Their food intake was monitored, and body weight measurements were obtained every second day.

X-ray computed tomography (CT) scanning

Eighty-two days after starting the experimental diet, the body composition (fat and muscle amounts) and bone mineral contents, bone volume, and bone mineral density (BMD) were measured using an X-ray CT system for small experimental animals with a rat mode (LaTheta LCT-100, Aloka, INC., Tokyo, Japan) [16]. The visceral and subcutaneous fat volumes computed automatically were compared with those after the radiologist's adjustments. Ratios of volumetric visceral fat-to-total fat and visceral fat-to-subcutaneous fat were compared on average and with single-slice measurements obtained at L4 and L5 vertebral body levels. The visceral and subcutaneous fat volumes were computed on the tomographic scanning images at 1.5-mm intervals in the measurement area presented with 2 yellow lines in Fig. 1A.

Bone parameters (bone mineral contents, bone volume, and BMD) were computed on the tomographic scanning images at 1.0-mm intervals in the measurement area between the proximal and distal epiphyses of the right femur [17]. The minimum cross-sectional moment of inertia and polar moment of inertia that represent the flexural rigidity and torsional rigidity, respectively, were also calculated automatically employing the software provided with the device. According to the manufacturer, the precision error (as % CV) was within 2% range for all measurements.

The length of the femur and the width of the femur were measured using a dial caliper, and the fresh weight of femur was measured. Then, the femurs were dried at 95 °C for 24 h to measure the dry weight of the femur. All the femurs were burnt to ash at 550–600 °C for 24 h, and the ash weight was measured. The ashed bone was dissolved in 1 M nitric acid to determine the calcium and phosphorus content in the femur.

Biochemical analysis of serum

Eighty-five days after starting the experimental diet, the animals were fasted overnight and sacrificed by bleeding from the abdominal aorta under anesthesia. Blood was collected and centrifuged at 2500 rpm for 15 min to extract the serum. Sera were collected and stored at −80 °C until being thawed for analyses.

Calcium was measured employing the o-cresol-phthalein complexion color development method [18], and inorganic phosphorus was determined using the method of p-methylaminophenol reduction [19]. Total amounts of protein were measured applying the Biuret method [20]. Alkaline phosphatase was determined employing the method of Bessey et al. [21]. Cross-linked N-teleopeptide of type I collagen (NTx) was measured using an enzyme-linked immunosorbent assay (Mochida Seiyaku Co., Ltd., Tokyo, Japan). Serum glucose, triglycerides, and total cholesterol were assayed using the glucose-enzyme [22], GK-GPO (glycerokinase-glycerol-3-phosphate oxidase) [23], and enzymatic determination [24] methods, respectively.

Serum growth hormone (GH) was measured employing the immuno-radiometric assay (TFB Inc., Tokyo, Japan). Insulin-Like Growth Factor-1 (IGF-1) was measured using the immuno-radiometric assay (Mitsubishi Kagaku Yatoron Inc., Tokyo, Japan). Insulin-Like Growth Factor Binding Protein 3 (IGFBP-3) was measured by radioimmunoassay (Bioclone Australia Pty Ltd., Sydney, Australia).

Statistical analysis

Values are shown as the mean ± standard error (S.E.). Comparisons between treatments (Cont. vs. PK, Cont. vs. MK) were conducted using the unpaired Student's t-test. Differences were considered significant at $p<0.05$. Analysis was conducted using SPSS17.0J (SPSS Inc., USA).

Results

Animals and diets

There were no significant differences in the final body weight at 85 days among the Cont. (mean ± S.E., 287.3 ± 7.0 g), PK (272.0 ± 3.7 g), and MK (281.5 ± 3.7 g) groups. In addition, there were no significant differences in the food intake (g/day) among the Cont. (mean ± S.E., 15.6 ± 0.3 g/day), PK (15.2 ± 0.4 g/day), and MK (16.2 ± 0.2 g/day) groups. No significant differences among the three groups (Cont., PK and MK) in the body weight gain (g/day) or food efficiency (body weight gain/food intake) were noted (data not shown). The vitamin K diets contained 0.06% PK or MK-4, respectively. Therefore, the amount of vitamin K intake from the experiment diets was calculated approximately 9–10 mg/day (30–35 mg/kg body weight).

Bone mass and bone mineral density of femur

As shown in Table 1, the width of the femur in the MK group was significantly higher than in the Cont. group ($p<0.05$). The dry and ash weights of the femur in the MK group were also significantly higher than those of the Cont. group ($p<0.05$, respectively). There was no significant difference in the width, dry weight, and ash weight of the femur between the Cont. and PK groups.

Moreover, the addition of MK-4 led to a significant increase in the total, cortical, cancellous, and trabecular volumes of the femur in the MK group compared with the control group ($p<0.01$, $p<0.05$, $p<0.01$, and $p<0.05$, respectively, Table 2). There was no significant difference in the total, cortical, cancellous, and trabecular volumes of the femur between the Cont. and PK groups.

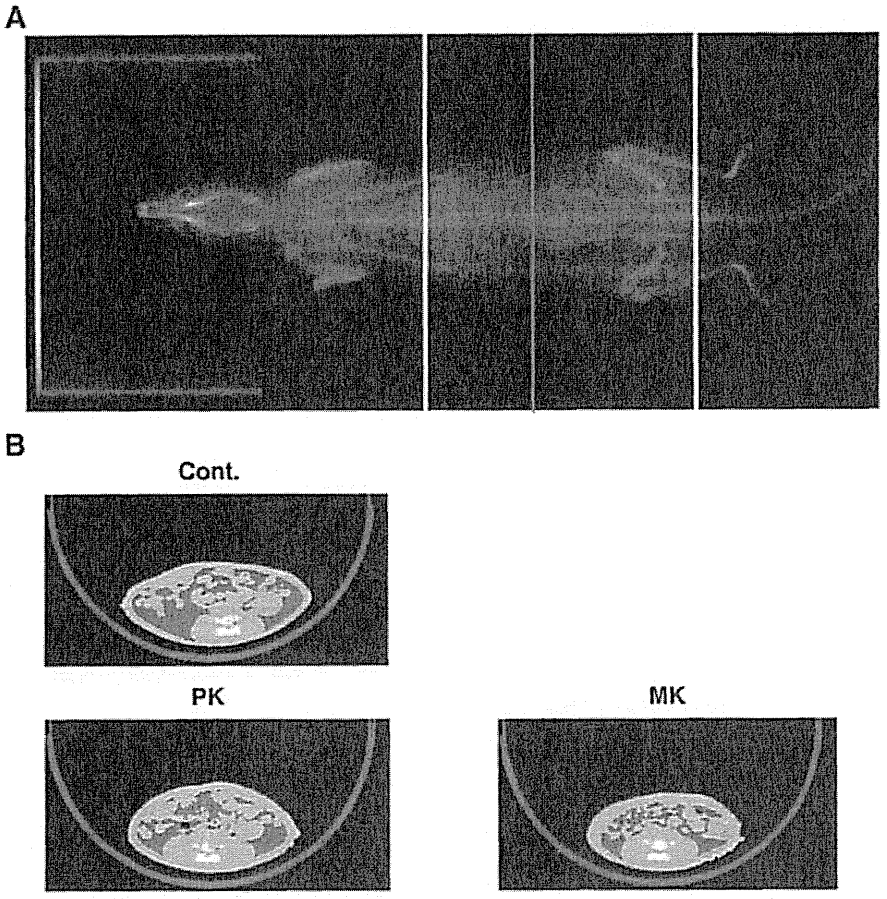

Fig. 1. X-ray computed tomography (CT) scanning after 82 days on the control (Cont.), PK (PK), or MK (MK) diet. (A) Representative images on X-ray CT scanning of the whole bodies of rats. For body composition measurements, tomographic images were acquired at 1.5-mm intervals in the measurement area presented as 2 yellow lines. (B) Cross-sectional appearance of rats in the Cont., PK, and MK groups. Tomographic X-ray CT images of the same 4th lumbar vertebral regions shown with a red line in panel A. The areas indicated in pink, yellow, and light-blue are visceral fat, subcutaneous fat, and muscle, respectively.

As shown in Table 3, the total bone mineral content (BMC) of the femur was higher in the PK and MK groups than that in the Cont. group ($p<0.05$ and $p<0.01$, respectively). The cortical, cancellous, and trabecular BMC in the MK group were significantly higher than those in the Cont. group ($p<0.05$, respectively, Table 3).

The BMD of the femur is shown in Fig. 2A. The BMD of the PK group was significantly higher than that of the Cont. group ($p<0.05$).

Bone strength

Two types of bone strength parameters: the minimum moment of inertia and polar moment of inertia of cross-sectional areas of the femur, are shown in Figs. 2B and C, respectively. The former parameter represents the flexural rigidity, and the latter torsional rigidity. Both of the minimum moment of inertia and polar moment of inertia of cross-sectional areas of the femur were significantly higher in the MK than in the Cont. group ($p<0.05$, respectively, Figs. 2B and C).

X-ray CT scanning of the fat area

Fig. 3 shows the results for the fat pad (g) after 82 days in the Cont., PK, and MK groups using an X-ray CT system for laboratory animals. The total fat weights were significantly lower in the PK and MK groups than in the Cont. group ($p<0.05$) (Fig. 3A). In the PK group, the weight of visceral fat was significantly lower than in the Cont. group ($p<0.01$, Fig. 3B). In the MK group, both the weights of visceral and subcutaneous fat were significantly lower than in the Cont. group ($p<0.05$, respectively, Figs. 3B and C). There was no significant difference in the amount of muscle among the Cont. (mean ± S.E., 97.9 ± 2.1 g), PK (101.4 ± 2.5 g), and MK (104.4 ± 2.2 g) groups.

Table 1
Length and weight of femur.

Groups	Length			Weight			
	Length (cm)	Width (cm)	Thickness (cm)	Fresh weight (g)	Dry weight (g)		Ash weight (g)	
Cont.	3.48 ± 0.02	0.35 ± 0.01	0.28 ± 0.01	0.810 ± 0.018	0.534 ± 0.010		0.357 ± 0.008	
PK	3.53 ± 0.03	0.35 ± 0.00 #	0.28 ± 0.01	0.830 ± 0.013	0.554 ± 0.010 #		0.374 ± 0.006 #	
MK	3.51 ± 0.02	0.39 ± 0.01	0.29 ± 0.00	0.863 ± 0.019	0.576 ± 0.010		0.384 ± 0.007	

Each value represents mean ± S.E.
#: Significant difference between the value of the control group and the MK group (#: $p<0.05$).

Table 2
Femur volume.

	Total volume (cm^3)		Cortical volume (cm^3)		Cancellous volume (cm^3)		Trabecular volume (cm^3)	
Cont.	0.493 ± 0.010		0.367 ± 0.007		0.126 ± 0.004		0.090 ± 0.003	#
PK	0.507 ± 0.010	##	0.380 ± 0.006	#	0.127 ± 0.005	##	0.098 ± 0.005	
MK	0.533 ± 0.008		0.393 ± 0.005		0.141 ± 0.005		0.105 ± 0.005	

Each value represents mean ± S.E.
#: Significant difference between the value of the control group and the MK group (#: $p<0.05$, ##: $p<0.01$).

Biochemical analysis of serum parameters

There were no significant differences in the levels of serum total protein, calcium, inorganic phosphorus, ALP, NTx, GH, IGF-1, IGFBP-3, and glucose among the three groups (Tables 4 and 5, Fig. 4A). Interestingly, the levels of serum triglycerides were significantly lower in PK and MK groups than in the Cont. group ($p<0.05$ and $p<0.01$, respectively) (Fig. 4B). In addition, the level of serum total cholesterol was significantly lower in the MK group than in the Cont. group ($p<0.05$).

Discussion

We compared the effect of PK or MK-4 on BMD, bone strength, fat accumulation, and serum parameters *in vivo*. The total BMC and BMD of the femur were significantly increased after 82 days on the PK compared to the Cont. diet ($p<0.05$, respectively) (Table 3 and Fig. 2A). As shown in Tables 1 and 2, the width, dry or ash weight, and total volume of the femur in the MK group were significantly higher than those of the control group ($p<0.05$, $p<0.05$ and $p<0.001$, respectively). Further, significant increases in the BMC, minimum moment of inertia of cross-sectional areas, and polar moment of inertia of cross-sectional areas of femur were observed in the MK group ($p<0.05$, respectively) (Table 3 and Figs. 2B and C).

In the present study, we revealed the different effects of PK or MK-4 on femoral bone parameters (BMD, width, dry weight, ash weight, total volume, minimum moment of inertia of cross-sectional areas, and polar moment of inertia of cross-sectional areas). As shown in Fig. 2A, BMD of the femur was significantly higher in the PK group, whereas BMD of the femur was not significantly higher in the MK group. In the MK group, femoral bone parameters (dry weight, ash weight, total volume and BMC) were significantly increased (Tables 1, 2 and 3). Femoral BMD was calculated per cm^3 (bone volume), so we considered the significant increase of bone volume as the reason why femoral BMD was not significantly higher in the MK group. Moreover, it will be also understood that the significant increase of bone volume was one of the reasons why femoral bone strength parameters (minimum moment of inertia of cross-sectional areas, and polar moment of inertia of cross-sectional areas) were significantly higher in the MK group. In addition, the increase of bone volume seemed to be caused by the increase of bone width not by the increase of bone length (Table 1). These results suggested that PK has the beneficial effects on increasing both of femoral BMC and BMD, while MK has the beneficial effects of increasing femoral BMC, bone volume, width and bone strength parameters. It is interesting that the effect of MK in the growth process on the width of the bone was suggested during bone remodeling.

Several *in vitro* studies demonstrated that both PK and MK-4 have beneficial effects on bone formation [25–27]. It was reported that MK-4 suppressed bone resorption [28–30], but PK did not have such an effect [31]. Hara et al. reported that the inhibitory effect of MK-4 on bone resorption may not be due to γ-carboxylation and that the side chain of MK-4 may play an important role in this inhibitory effect [31]. MK-4 significantly inhibited calcium release from mouse calvaria treated with $1\alpha,25(OH)_2D_3$ or prostaglandin E_2 (PGE_2), and the inhibitory effect of MK-4 on calcium release from calvaria was not affected by the addition of warfarin, an inhibitor of the vitamin K cycle while PK at the same doses did not have these effects [31]. Therefore, the inhibitory effect of MK-4 on bone resorption does not seem to be via γ-carboxylation.

A previous study demonstrated that MK-4 inhibited decreasing bone strength measured by employing a 3-point bending test induced by ovariectomy [15]. Bone quality has become an important issue in the prevention of osteoporosis [32], because the BMD is not the only factor that affects the occurrence of fractures [33,34]. The NIH consensus meeting proposed that bone strength is related to many factors including bone mineralization, architecture, turnover, and the concentration of organic proteins [35]. Recent studies have revealed that vitamin K functions as a ligand for nuclear steroid and xenobitoic receptor (SXR), as well as a cofactor for γ-carboxylase [36]. Inoue et al. identified novel SXR target bone-related genes regulated by MK-4 in osteoblastic cells using microarray analysis. Among extracellular matrix-related genes, they revealed that a small leucine-rich repeat proteoglycan, tsukushi, contributed to collagen accumulation [37].

Recently, we reported for the first time that both PK and MK-4 as nutritional factors enhance intestinal alkaline phosphatase (ALP) activity [38]. The high activity of intestinal ALP, which localizes at the brush border of the intestinal epithelium cells, suggests the participation of this enzyme in the transport of nutrients. In humans and rodents, a diet with a high fat content or the fat-feeding had elevated serum levels of intestinal ALP activity [39,40]. It was reported that intestinal ALP knockout mice showed no difference from the wild-type controls under the normal chow, however, when maintained long-term on a high-fat diet, the intestinal ALP knockout mice showed faster body weight gain

Table 3
Bone mineral measurements of femur.

	Total BMC (mg)		Cortical BMC (mg)		Cancellous BMC (mg)		Trabecular BMC (mg)	
Cont.	304.5 ± 7.2	*	248.1 ± 5.9		56.4 ± 1.6		48.1 ± 1.6	
PK	326.3 ± 5.9		265.6 ± 4.8		60.7 ± 3.1		53.6 ± 3.1	
MK	339.7 ± 7.9	##	272.7 ± 5.0	#	66.9 ± 3.1	#	58.7 ± 3.1	#

Each value represents mean ± S.E.
: Significant difference between the value of the control group and the PK group (: $p<0.05$).
#: Significant difference between the value of the control group and the MK group (#: $p<0.05$, ##: $p<0.01$).

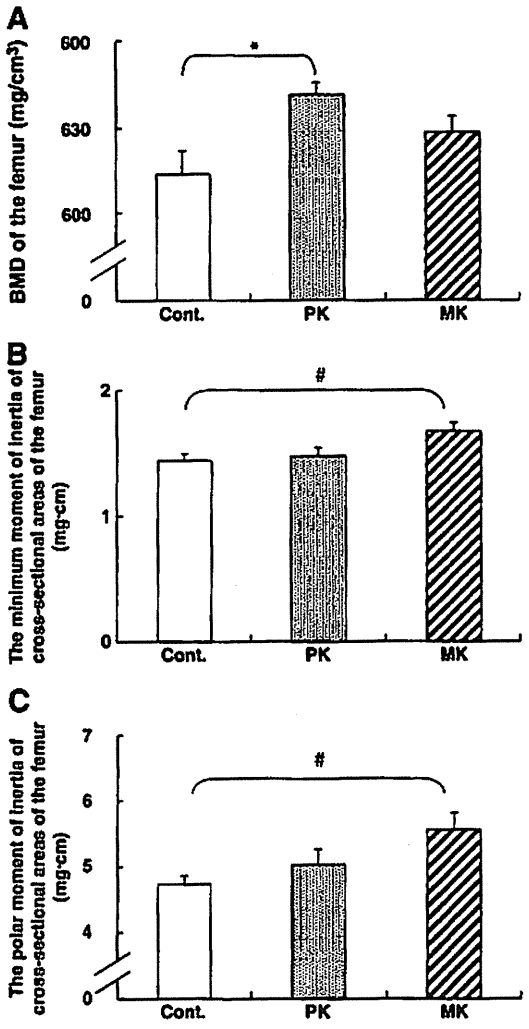

Fig. 2. Bone mineral density (BMD) and bone strength parameters of the femur in the control (Cont.), PK (PK), or MK (MK) diet group. Results are the mean ± S.E. of 7 or 8 animals. Significant difference between the PK and Cont. groups (*: $p<0.05$). Significant difference between the MK and Cont. groups (#: $p<0.05$). (A) BMD of the femur. (B) The minimum moment of inertia of cross-sectional areas of the femur. (C) The polar moment of inertia of cross-sectional areas of the femur.

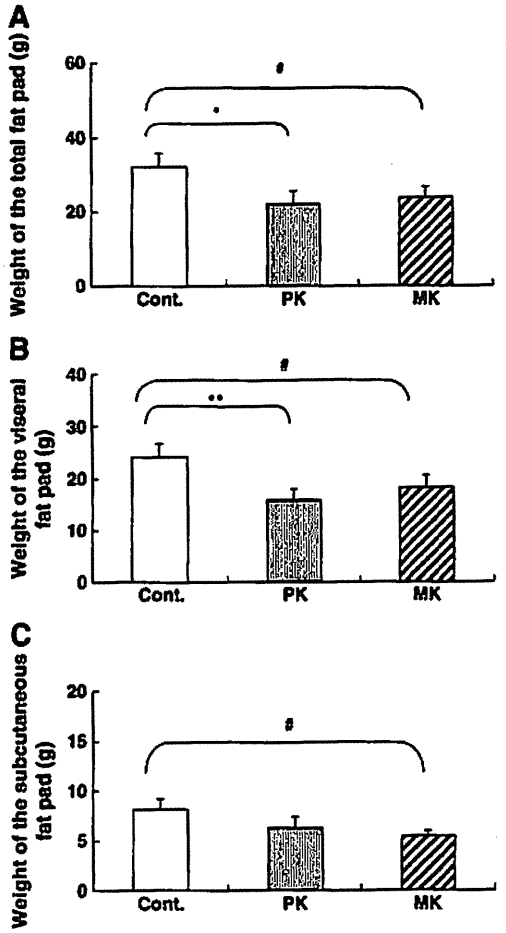

Fig. 3. Weight of the fat pad. (A) Weight of the total fat pad (g), (B) weight of the visceral fat pad (g), and (C) weight of the subcutaneous fat pad (g) after 82 days on the control (Cont.), PK (PK), or MK (MK) diet. Results are the mean ± S.E. of 7 or 8 animals. Significant difference between the PK and Cont. groups (*: $p<0.05$, **: $p<0.01$). Significant difference between the MK and Cont. groups (#: $p<0.05$).

compared with the wild-type animals [41]. These finding suggests the possibility that intestinal ALP may regulate not only phosphate metabolism but also fat metabolism.

In order to examine whether the effect of MK-4 on the bone volume was via GH secretion, we investigated the level of GH in serum [42–44]. Although the level of GH in the MK group tended to be higher than in the Cont. group, there was no significant difference, as shown in Table 5. In addition, we measured the levels of IGF-1 and IGFBP-3 in serum, which are markers of bone-related growth. As the results, IGF-1 and IGFBP-3 were similar among the three groups (Cont., PK, and MK) (Table 5), and the supplementation of MK-4 did not influence these growth factors affecting bone metabolism.

Interestingly, the addition of both PK and MK-4 to the Cont. diet may regulate not only bone strength but also fat deposition. Body weight gain (g/day), food intake (g/day) and food efficiency (body weight gain/food intake) were not significantly different among the three groups (Cont., PK, and MK). As shown in Fig. 3A and B, the weights of total and visceral fat in both the PK and MK groups were significantly lower than in the Cont. group. A previous *in vitro* study reported that MK-4 but not PK inhibited the formation of adipocytes in bone marrow cells [45]. It demonstrated that MK-4 inhibited the expression of osteoclast differentiation factor (ODF)/RANK ligand and the formation of osteoclast-like cells induced by $1\alpha,25(OH)_2D_3$, and that MK-4 specifically influenced the differentiation and functions of bone marrow cells. The present study revealed that both PK and MK-4 had effects on the reduction of fat deposition *in vivo*. Structural differences in the isoprenoid side chain may influence vitamin K metabolism, including the way it is transported, taken up by target tissues, and subsequently excreted. In the post-prandial state, PK is transported mainly by triglyceride-rich lipoproteins (TRL) and long-chain MKs mainly by low-density lipoproteins (LDL) [46]. As shown in Fig. 4B, the levels of triglycerides in both PK and MK groups were significantly decreased ($p<0.05$ and $p<0.001$, respectively). PK is converted into MK-4 and accumulates in extrahepatic tissues [47], and so we suggest that the regulation of fat deposition might be

Table 4
Biochemical parameters of serum.

Groups	Total protein (g/dl)	Calcium (mg/dl)	Phosphorus (mg/dl)	Alkaline phosphatase (U/l)	NTx (nmol/l)
Cont.	7.1 ± 0.1	10.6 ± 0.1	5.4 ± 0.6	118.7 ± 6.6	11.9 ± 0.5
PK	6.9 ± 0.1	10.4 ± 0.1	5.6 ± 0.5	136.1 ± 11.2	12.9 ± 0.8
MK	7.0 ± 0.2	10.5 ± 0.1	6.6 ± 0.4	125.6 ± 13.9	12.4 ± 0.9

Each value represents mean ± S.E.

Table 5
Hormone and cytokine parameters of serum.

Groups	GH (ng/ml)	IGF-1 (ng/ml)	IGFBP-3 (μg/ml)
Cont.	0.009 ± 0.001	1.92 ± 0.23	0.145 ± 0.007
PK	0.006 ± 0.002	1.69 ± 0.13	0.144 ± 0.003
MK	0.041 ± 0.017	1.43 ± 0.18	0.158 ± 0.006

Each value represents mean ± S.E.

mediated by not only dietary vitamin K, but also MK-4 converted from PK.

Some recent studies proposed that osteocalcin of undercarboxylated form is involved with a hormone in the endocrine regulation of energy homeostasis [48], and that picomolar amount of undercarboxylated osteocalcin regulates the expression of insulin genes and beta-cell proliferation markers whereas nanomolar amounts of osteocalcin affects adiponectin expression [49].

The effect of vitamin K on fat mass could be mediated through adiponectin regulation which itself has been found to be associated with fat mass. There is also another recent published work in humans that vitamin K supplementation with a daily dose of 0.5 mg of phylloquinone for 3 years had a protective effect on the progression of insulin resistance in older men [50]. These data indicate the need for further research and better understanding of the relationship among osteocalcin, its carboxylation, and vitamin K intakes.

The amount of vitamin K intake from the experiment diets is massive compared to nutritional requirements for vitamin K, and

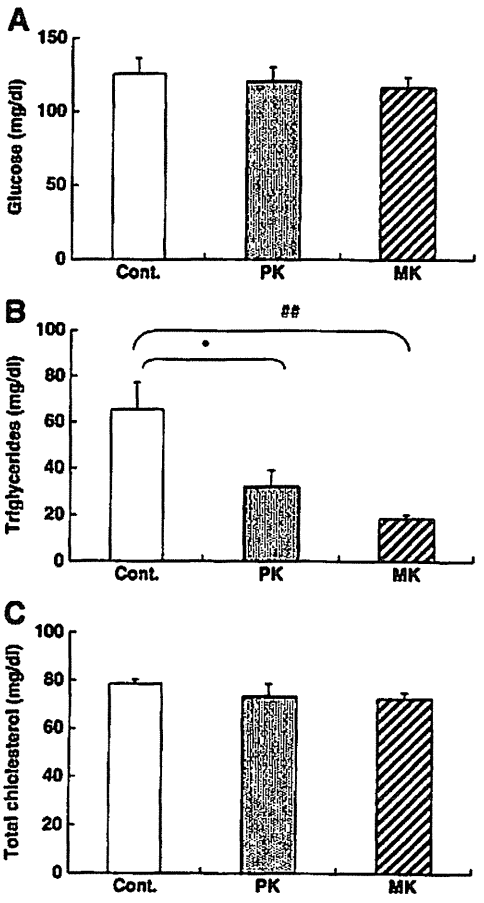

Fig. 4. Levels of serum glucose (A), serum triglycerides (B), and serum total cholesterol (C) after 85 days on the control (Cont.), PK (PK), or MK (MK) diet. Results are the mean ± S.E. of 7 or 8 animals. Significant difference between the PK and Cont. groups (*: $p<0.05$). Significant difference between the MK and Cont. groups (##: $p<0.01$).

further dose–response studies are required to investigate whether long-term supplementation with doses in a more nutritional range would deliver the changes seen in this study.

Further studies on the effects of vitamin K on the regulation of the body composition would provide useful data on the prevention of lifestyle-related disorders, including osteoporosis.

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Association of CYP19 Gene Polymorphism with Vertebral Fractures in Japanese Postmenopausal Women

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Association of *CYP19* Gene Polymorphism with Vertebral Fractures in Japanese Postmenopausal Women

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Abstract This study investigates aromatase gene polymorphism, which might influence bone strength in terms of mineral density and quality. We explored the relationship between *CYP19* polymorphisms and vertebral fractures in postmenopausal Japanese women. In addition, we compared estrogen and testosterone levels in Japanese postmenopausal women with and without fractures. Osteoporotic postmenopausal women showed higher incidences of vertebral fractures than osteopenic women or women with normal lumbar bone mineral density (L2-4 BMD). Estrogen concentrations in postmenopausal women were associated with BMD; however, no association was found between sex hormone levels and the presence of fractures. The C allele rs2470152 was significantly associated with increased risk of vertebral fractures ($P = 0.04$), whereas none of the *CYP19* polymorphisms showed differences in sex steroid levels between subjects with and without fractures. Allelic variants of aromatase genes appear to interact to influence the risk of vertebral fractures in postmenopausal Japanese women.

Keywords Aromatase gene polymorphisms · Vertebral fractures · Postmenopausal women

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Introduction

Osteoporosis is caused by multiple factors, including environmental factors (such as calcium intake), exercise, and estrogen levels. The main source of estrogen in postmenopausal women is the aromatization of androgenic precursors, a reaction catalyzed by the cytochrome P450 (CYP) aromatase enzyme, encoded by *CYP19* located on chromosome 15q21.1. It has recently been reported that estrogen levels are genetically determined by aromatase activity (Olson et al. 2007; Haiman et al. 2007; Sowers et al. 2006). In addition, allelic variants of the aromatase gene have been associated with bone mineral density (BMD) and bone fractures (Hong et al. 2007; Masi et al. 2001; Somner et al. 2004). A/G polymorphisms in the 3′ untranslated region (UTR) and the I.2 promoter (rs10046 and rs1062033; Rinancho et al. 2005) and an A/G polymorphism in the I.6 promoter rs4775936 (Enjuanes et al. 2006) of the aromatase gene have been studied in relation to osteoporosis and BMD, but the results remain controversial. In addition, an rs2470152 polymorphism in the aromatase gene has been shown to affect serum estrogen levels in Swedish men (Eriksson et al. 2009). Therefore, in order to analyze the association with the risk of vertebral fractures in postmenopausal women, we conducted a cross-sectional study of the interaction between *CYP19* gene polymorphisms and sex steroid hormone levels or risk of vertebral fractures in Japanese postmenopausal women. In this study, we focused on four markers (rs2470152, rs4775936, rs1062033, and rs10046) to clarify the association between polymorphisms in aromatase genes and vertebral fractures.

Materials and Methods

Study Subjects

Three hundred sets of genomic DNA and serum samples were provided from the collected samples of the Institute of Medical Sciences, Tokyo University, obtained for tailor-made medicine realization projects. These samples were collected from the various institutions that were members of these projects following the approval of the individual ethics committees. Ethical approval was obtained from the Ethics Committee of the Leading Project for Personalized Medicine in the Institute of Medical Science, University of Tokyo, and the Tokyo Metropolitan Geriatric Hospital. Another 300 DNA samples were collected from women for the purpose of analyzing the relationship between polymorphisms and the etiology of disease in the Japanese population. The samples were provided by the Leading Project for Personalized Medicine of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

The samples were divided into three categories according to the *T* score of the measurement of lumbar spine BMD (L2-4 BMD) by dual energy X-ray absorptiometry (DXA) as defined by the World Health Organization: *T* scores of −1.0 and above were classified as normal BMD, scores of −2.5 to −1.0 were considered osteopenia, and scores below −2.5 were considered osteoporotic.

For assessment of vertebral fractures, anteroposterior and lateral X-ray examinations of the thoracic and lumbar spine were performed. Morphometrically,