A. 握力：サルコペニアの診断と治療の目標のうち最も重要な移動能力低下に直結するのはもちろん下肢筋力であるが、後述するように下肢筋力の簡便かつ正確で十分に普及した測定法はまだない。一方、測定器が安価で普及している握力は、下肢筋力や膝伸展トルク、筋断面積によく関連し、加えて、移動等の予後予測因子として筋量より優れているとされる[15]。縦断調査での握力とADL低下の間には直線的な関連がみられた[16]。握力は、筋力の研究と臨床において簡便で良好な方法で、下肢筋力と良好な相関を有するとされる[6]。

B. 膝伸展屈曲力：膝関節の伸展屈曲力は、下肢筋力の代表部位で、多くの種類の精密測定機器が販売されている。等運動性収縮で測定された膝伸展力は、ADLに特に関連するとされ、70－80歳代の膝筋力は20－40歳代に比べて20－40%減少するとされる[17]。膝伸展屈曲力は、筋力の研究において有力な方法であるが、臨床においては特別な測定器と訓練が必要なため限界がある[6]。

6. 身体機能評価法

身体機能の評価結果には、やる気などの他要因が筋力測定以上に関与し、検査法にも大変多くの種類があるので、どれを選択すべきかなどに定まった見解はないが、身体的自立に直結する身体機能を評価することは非常に重要である。

A. Short Physical Performance Battery (SPBB)：この評価法は、我が国ではまだあまり知られていない。老年医学で使われている指標で、両足をそろえた位置やタンデムポジションによる立位でのバランス能力、4m歩行時間、椅子からの立ち上がり5回の時間の3つのパフォーマンスを調べ、0から12点までの点数で評価する方法である[18]。SPBBは、身体機能の研究と臨床においてはスタンダードの方法とされる[6]。

B. 通常歩行速度：加齢とともに低下する歩行速度は、歩調と歩幅で規定され、高齢で生じる歩幅の急速な減少に大きく影響される。この歩幅減少は、脚長の減少に加えて、膝関節の最大伸展が接地前に終了して屈曲位で接地するために起こる。そして、通常歩行速度は下肢筋力と強い関連性を有しており[19]、機能障害発生の予測因子である[18]。通常歩行速度は、身体機能の研究と臨床においては、SPBBにも含まれている項目であるが、これだけでも使用可能な方法とされる[6]。

C. Timed up and go test(TUG)：3m TUGは、我が国の運動器不安定症の診断基準で取り上げられている動的バランスを評価できる方法で、椅子に座った姿勢から立ち上がり、3m先の目印でターンして椅子に座るまでの時間である。3m TUGは、身体機能評価法として使用可能な方法とされる[6]。

7. サルコペニア診断のカットオフ値とアルゴリズム（図2と表3）

筋量、筋力、身体機能の各評価法のうち、前述したように信頼性や実用性等から臨床での使用を薦められるものとして、DXAやBIAによる補正四肢筋量、握力、SPPBや歩行速度が上げられ、それぞれのカットオフ値を表3に示した。

欧州合意では、診断の手順として、まず、身体機能からスクリーニングを開始して身体

機能低下がなければ筋力を評価し、それも正常ならサルコペニアなし、低下あれば筋量測定、また、身体機能低下があればやはり筋量を評価し、筋量が正常ならサルコペニアなし、低下があればサルコペニアと診断するというアルゴリズムが提示された（図2）。その際、身体機能はSPPBではなく、歩行速度が推奨され、カットオフ値は0.8m/sと明記された。筋力は握力、筋量は補正四肢筋量で判定するが、カットオフ値はアルゴリズムには記載されず、表3のように別記された。このなかのどれを選択するかについての明確な示唆はなく、特に日本人にこのまま当てはめることにはまだ十分な議論が必要と考えられる。

8. 自験例での解析

DXAによる補正四肢筋量の解析で、Baumgartnerのカットオフ値と、ごく最近発表されたSanadaによる日本人の若年成人平均値－2標準偏差[20]の値を使用した場合に生じる違いを我々の施設で過去に測定した臨床例2868例の結果から示す（表4）。全体では、男女とも差は大きく、特に女性で顕著であった。我が国での健常若年成人データのさらなる集積による基準値の確立が必要と思われた。

表3　筋量、筋力、身体機能の薦められる測定法とカットオフ値（文献6から作成）

	測定法	カットオフ値	国
筋量	DXAによる補正四肢筋量*	男性：7.26 kg/m^2 女性：5.45 kg/m^2	米国
	BIAによる補正四肢筋量	男性：8.87 kg/m^2 女性：6.42 kg/m^2	台湾
筋力	握力	男性：30 kg 女性：20 kg 男性：BMI ≦24: 24kg BMI 24.1-26：30kg BMI 26.1-28：30kg BMI >28：32kg 女性：BMI ≦23：17kg BMI 23.1-26：17.3kg BMI 26.1-29：18kg BMI >29：21kg	イタリア 米国
身体機能	Short Physical Performance Battery(SPBB)	SPPB ≦8	米国
	歩行速度	6mコース：1 m/s 1.175m/s 15フィートコース: 男性：身長≦173 cm：0.65 m/s 身長>173 cm：0.76 m/s 女性：身長≦159 cm：0.65 m/s 身長>159 cm：0.76 m/s 4mコース：0.8 m/s	米国 米国 米国 イタリア

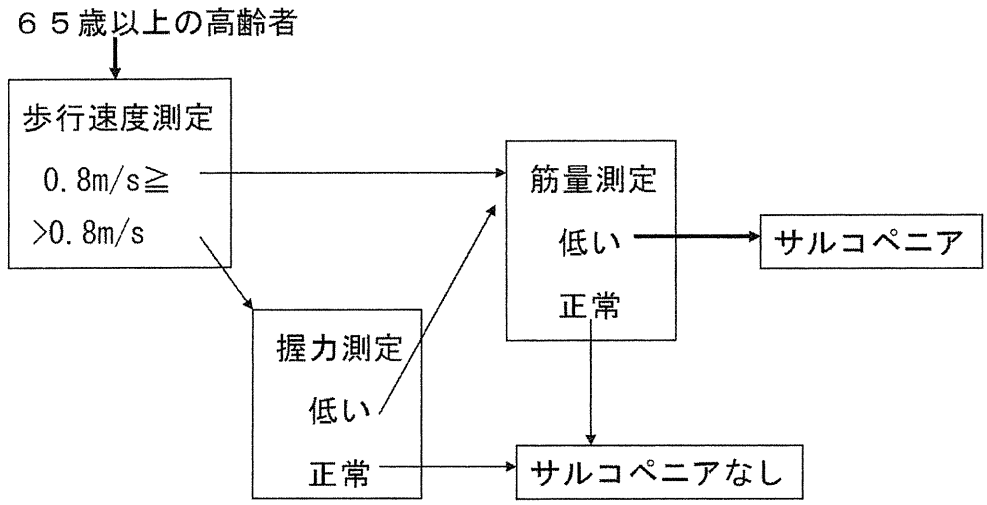
＊日本人参考値 男性: 6.87 kg/m^2、女性: 5.46 kg/m^2（文献20による）

9. 診断に関する今後の課題

これまでサルコペニアの診断に関する現状を述べて来た。サルコペニアの医学が本当に高齢社会に役立つように発展するかどうかは、これからにかかっている。

現在の大きな課題は、骨粗鬆症に対応させると理解しやすい。骨粗鬆症の診断と治療の最終目標として骨折という明瞭なイベントに定めることができたため、この20年間で大きな発展を遂げ、高齢社会への実質的な貢献を実現することができた。一方、サルコペニアでは、何を最終目標とするかについては、欧州合意では、身体機能、筋力、筋量という評価項目そのものを主要アウトカムとすると示しているが、それで十分なのかは議論のあるところであろう。

日本で提唱されているロコモティブシンドロームは、最終目標が運動器障害による要介護化と明確になっており、この方が高齢社会には適合するのではないかとも思われる。一方、運動器不安定症という転倒リスクの高まった病態も既に保健収載されているが、その診断で使用される身体機能は、歩行速度ではなく、開眼片足立ち時間あるいは3 m TUGで



サルコペニアのスクリーニングのために使用することが示された。歩行速度や筋力の低下の原因疾患の鑑別は個々に考慮される必要がある。また、若年者にも応用できるとされた。

図2　サルコペニア診断の欧州合意によるアルゴリズム（文献6から改変作成）

表4　自験例における補正四肢筋量のカットオフ値の違いによるサルコペニアの診断率

	男性 (n=671)	女性 (n=2197)	全体
欧州基準による診断率	40%	78%	39%
日本人データによる診断率	55%	29%	36%

国立長寿医療研究センター整形外科臨床例をBaumgaertnerらの方法に沿って補正四肢筋量の評価のみで診断した結果

あり、その整合性の検討が急がれる。また、筋力評価部位は、簡便で安価で正確な機器さえあれば、もちろん下肢筋力で診断した方がよいことは自明であり、ニーズを満たす機器開発が急がれる。筋量は、それだけで筋の機能を表すには不足していることも明らかで、筋の質をCT、MRIを用いずとも簡便に評価する方法の研究進展が望まれる。

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医療の現場から

ロコモティブシンドローム

原　田　　敦*

内容紹介

ロコモティブシンドロームは，「運動器の障害による要介護の状態や要介護リスクの高い状態」と定義された．自己評価のためのロコモーションチェックでは，7項目（片足立ちで靴下がはけない，家のなかでつまずいたり滑ったりする，横断歩道を青信号で渡りきれない，階段を上るのに手すりが必要である，15分くらい続けて歩けない，2kg程度の買物をして持ち帰るのが困難である，家のやや重い仕事が困難である）のうち一つでも該当すればロコモと判定され，開眼片足立ち訓練，スクワット運動，太極拳やウオーキングなどのプログラムを中心としたロコモーショントレーニングにて自己訓練し，医療機関はその基礎疾患治療や専門的訓練指導等を行って，要介護化を防止する．

—Key words—
ロコモティブシンドローム，要介護化，運動器，予防

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はじめに

H19年の国民生活基礎調査によれば，65歳以上の高齢者における介護が必要になった原因として，脳血管疾患（23.3%），認知症（14.0%）などの主に脳の障害による病態と並んで，関節疾患（12.2%）や骨折・転倒（9.3%）などの主に運動器の障害による病態が21.5%と大きな割合を占めており，この傾向は特に女性で顕著である[1]．運動器とは，骨，軟骨，筋肉，腱，靭帯，神経などで構成される器官で，機能的には，骨と関節に大別され，脊椎が脊髄神経を収納する機能も備えている．高齢期におけるこれらの運動器の障害は，動物の生活に最も根本的な能力である“動く”に直接悪影響を及ぼすため，様々な程度の“動けない”という状態になり，それに応じたレベルの介護を受けないと生存できない状況がもたらされる．我が国は2007年に超高齢社会に突入しており，今後はますます運動器関連の要介護化の需要と供給が増加し続け，その傾向は後期高齢期でますます顕著になるものと予測される．

日本整形外科学会が，同じ2007年に以下に概説するロコモティブシンドロームを提唱したのは偶然ではなく，2011年の公益法人化の際の定

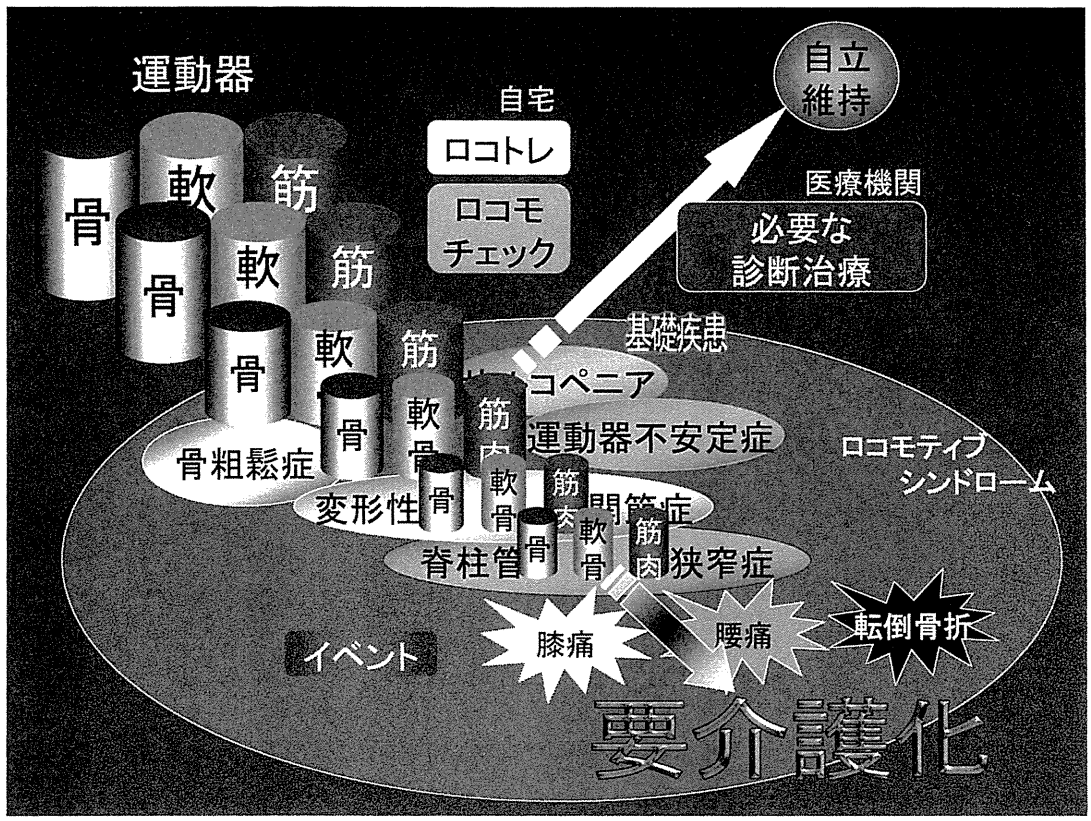

図1　ロコモティブシンドロームの概念（著者が自作）

款に整形外科学だけでなく，運動器学を学会の目的に明記したのも，このような社会の情勢に応えるためである．

Ⅰ．ロコモティブシンドローム（運動器症候群）の概念（図1）

前述したような「運動器の障害による要介護の状態や要介護リスクの高い状態」に対してロコモティブシンドロームという呼称を与え，別名を「運動器症候群」，略称を「ロコモ」として，中村耕三前理事長の主導により日本整形外科学会から提唱された[2]．この定義から分かるように，要介護化する前の発症リスク上昇に留まっているうちからセルフチェックにてスクリーニングして，軽いうちからセルフトレーニングで進行の予防を広く図り，運動器由来の要介護化を減らそうとするもので，症状が進んだ場合は，医療機関受診による原因疾患の診断と治療が必要である．

運動器を構成する三大組織は，骨，軟骨，筋肉で，これらはいずれも加齢とともに量的に減少し，質的に劣化することが知られている．その程度があるレベルを超えると，骨には骨粗鬆症，軟骨には変形性関節症，筋肉にはサルコペニア（筋肉減少症）など，移動や歩行などの重大な機能の低下をもたらす基礎疾患が多くは無症状のまま発生し，潜在的に運動機能を低下させ，要介護リスクを上昇させる．運動機能が後述するロコチェックに当てはまるようになればロコモティブシンドロームに該当することになる．また，さらに基礎疾患が進行すると，病態が表在化し，膝痛や腰痛，あるいは転倒骨折などのイベントが生じて，その程度が重いと，実際に介護を要する状態に陥ることになる．ちなみに，基礎疾患として上げたうち，運動器不安定症と脊柱管狭窄症は，関連領域が比較的限定

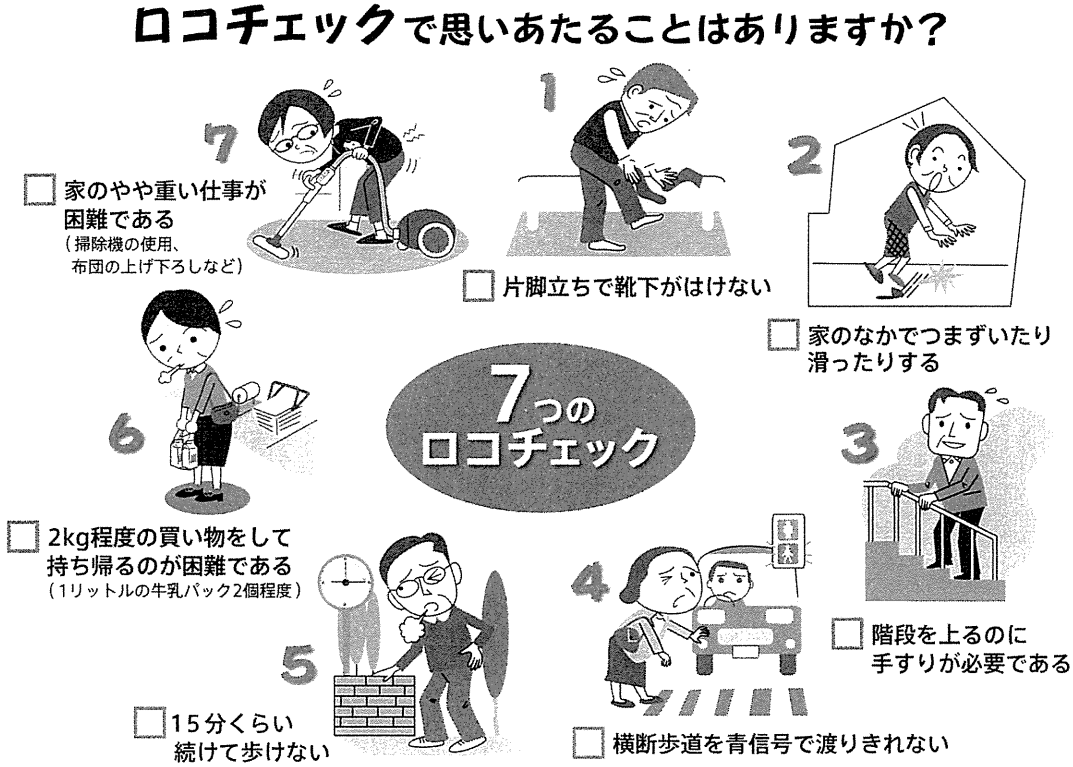

図2　ロコチェック
日本整形外科学会ホームページ「ロコモティブシンドロームとは」の「ロコモパンフレット（http://www.joa.or.jp/jp/public/locomo/locomo_pamphlet.pdf）」による．

された疾患であるが，頻度と重要性からは抜いて考えることはできないと思われる．前者は，サルコペニア等から由来する筋力・バランスの低下による転倒リスクの上昇で転倒骨折に関連し，後者は，変形性関節症で生じる脊柱管狭窄による疼痛や麻痺で歩行障害に関連する．

Ⅱ．ロコモーションチェック（ロコチェック）（図2）

これは医師が患者を診断するためのものではなく，中高年者が自宅などでロコモティブシンドロームの評価を気軽に行えるセルフチェック用のツールである．当初のチェック項目は，（1）片足立ちで靴下がはけない，（2）家のなかでつまずいたり滑ったりする，（3）階段を上るのに手すりが必要である，（4）横断歩道を青信号で渡りきれない，（5）15分くらい続けて歩けない，の5つであった．そのうちのひとつでも当てはまれば，ロコモティブシンドロームであるとされる．その後，ロコチェックに（6）2kg程度の買物をして持ち帰るのが困難である，（7）家のやや重い仕事が困難である，の2つの家事動作が追加され，全部で7つのうちひとつでも該当するとロコモと判定される．

国立長寿医療研究センター認知症先進医療開発センター予防開発部の疫学データによれば，握力，膝伸展力，片足立ち時間などは男女とも年齢とともに明らかに低下することが示されており[3]（図3），ロコチェックでは，これらの筋力・バランスを主体とした下肢機能低下が，簡

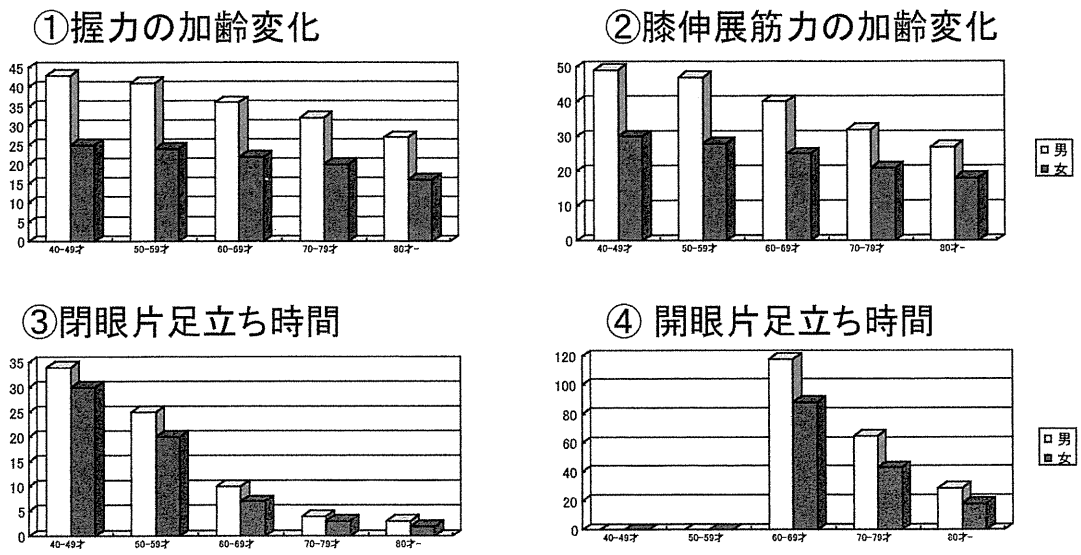

図3　筋力バランスの加齢変化
国立長寿医療研究センターホームページ　老化に関する長期縦断疫学研究　第6次調査　2008年7月～2010年7月（http://www.ncgg.go.jp/department/ep/monograph6thj/exercise.htm#1）から作成.

明かつ具体的な質問事項に巧みに取り入れられている.

Ⅲ. ロコモーショントレーニング（ロコトレ）（図4）

ロコチェックの結果，ロコモの可能性があれば，その改善や進行の予防のためのロコモーショントレーニングと呼ばれる運動訓練，ロコトレを開始する．もともとの個人の身体活動レベルに合わせて無理なく，安全に長く継続して行うことが基本であり，開眼片足立ち訓練，スクワット運動，太極拳やウオーキングなどの準スポーツ的プログラムなどが，薦められている.

それらのうちで基本訓練とされるのが，開眼片足立ちとスクワットで，自宅近くなら外出できる身体活動レベル以上の人では，左右1分ずつの両手を離した開眼片足立ちや両手を離したスクワット5－6回を一日3回行う．支えなく室内の移動ができるレベルの人では，片手を机などにつきながらの開眼片足立ちや両手を机につきながら，安全のために椅子を置くが腰掛けずにスクワットを行う．杖や伝い歩きなどで室内の移動ができるレベルの人では，両手を机などにつきながらの開眼片足立ちや両手を机につきながら，椅子に腰掛けてスクワットを行う．室内の移動はできないが，椅子に座ることができるレベルの人では，片足立ちは行わず，スクワットもできないときは，椅子に腰掛けて机に両手をつきながら腰を浮かす動作を行う.

Ⅵ. 医療機関の役割

上述したように，運動機能の低下をロコチェックで確かめ，ロコトレを自分で行っても，十分な改善が得られない，もっと有効な運動訓練を希望される，さらに腰痛や膝痛などの疼痛が出現して，その治療も希望される場合などでは，医療機関を受診することも少なくない．そこでは，医師は，機能低下の程度を医学的に確認すると同時に，基礎疾患の有無と重症度を適切に診断し，必要なら基礎疾患に対する治療を開始する．さらに，専門的な立場から安全かつ効率的な運動訓練指導とモチベーションを上げる工夫等を行う．また，疼痛があれば，その診断と治療を適格に加えて緩和させ，廃用性萎縮

などのロコモティブシンドロームの悪循環連鎖（ロコモティブドミノ）を絶って，高齢者の自立を維持させることが医療機関の重要な役割と思われる（図1）.

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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.

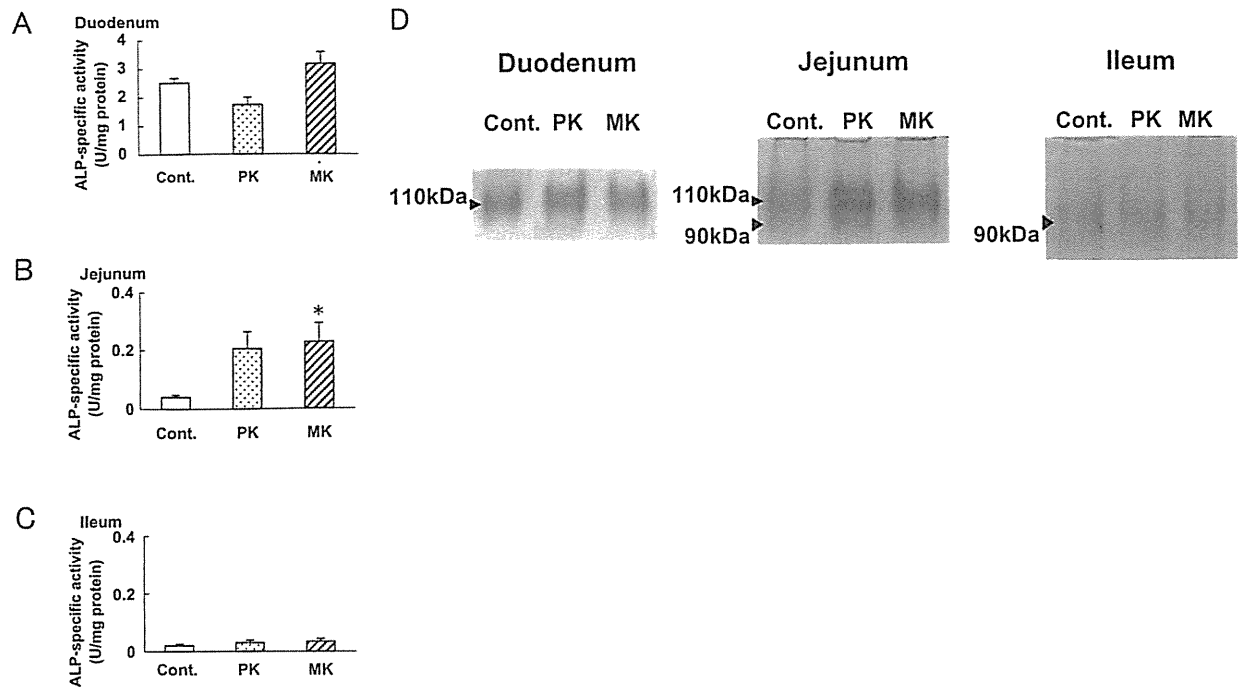

Fig. 1. ALP-specific activities of the duodenum (A), jejunum (B), and ileum (C). Results are the mean±SE of 7 animals. Significant difference between the MK and control groups (*$p<0.05$). D: Mouse intestinal ALP isozymes separated by polyacrylamide gel electrophoresis. The gels was stained for ALP activity 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

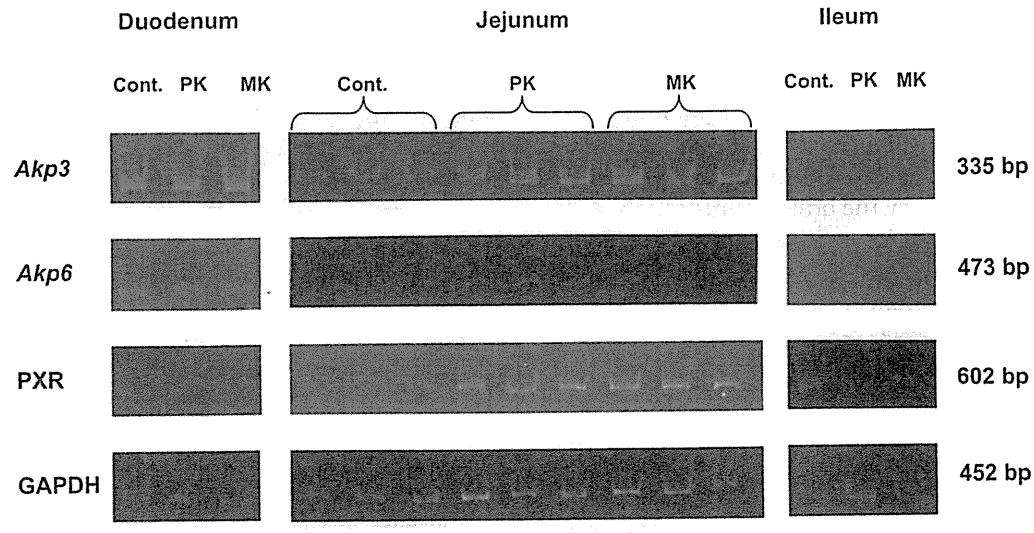

Fig. 2. Detection by RT-PCR of mRNAs for *Akp3*, *Akp6*, PXR, and GAPDH in the duodenum, jejunum, and ileum. PCR products were electrophoresed in a 5.25% polyacrylamide gel. Cont.: control, PK: phylloquinone, MK: menaquinone-4.

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).

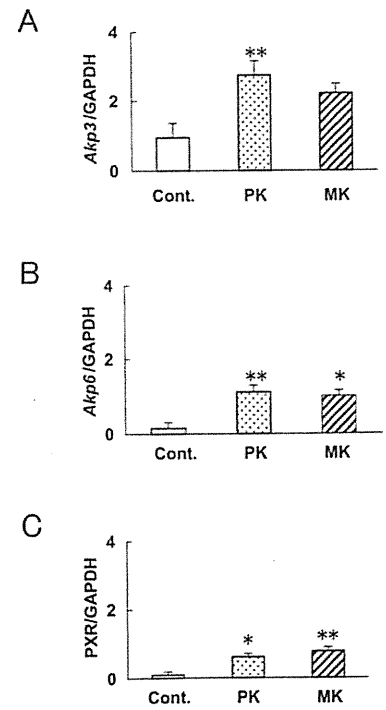

Fig. 3. The relative density of PCR products in the mouse jejunum. The diagrams show the relative density of the PCR products. A: The relative density of PCR products for *Akp3*. B: The relative density of PCR products for *Akp6*. C: The relative density of PCR products for PXR. Results are the mean±SE 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.

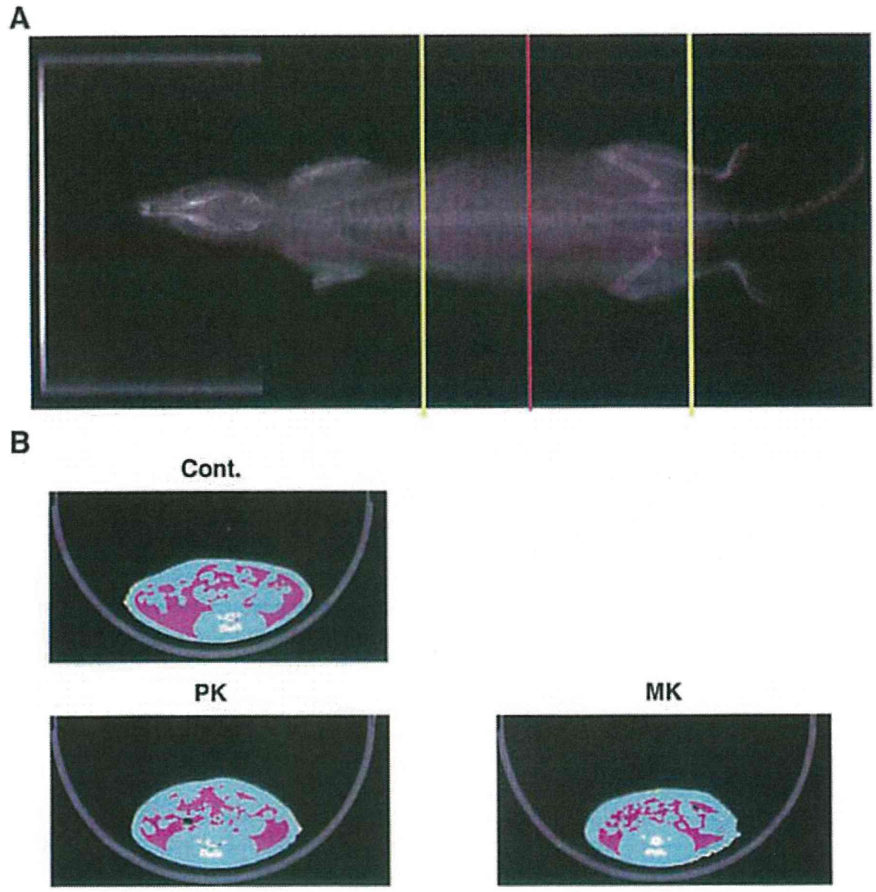

Fig. 1. X-ray computed tomography (CT) scanning after 82 days on the control (Cont.), PK (PK), or MK (MK) diet. (A) Representative images on X-ray CT scanning of the whole bodies of rats. For body composition measurements, tomographic images were acquired at 1.5-mm intervals in the measurement area presented 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 (#: $p<0.05$).