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(RESEARCH ARTICLE)

Roles of vitamin K (phylloquinone and menaquinones) and related proteins (prothrombin, des-γ-carboxyprothrombin, and γ-glutamic acid) in healthy adults

Yuichi Fukuzawa [1], Katsuhiro Gotoh [1] and Soichiro Uehara [2]

[1] *Pharmacy Department, Hijirigaoka Hospital, Hokkaido, Japan.*
[2] *Department of Gastroenteronology Hijirigaoka Hospital, Hokkaido, Japan.*

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Abstract

The aim of the study was to evaluate the plasma levels of vitamin K (VK), prothrombin, des-γ-carboxyprothrombin (DCP) by ELISA with two types of monoclonal antibodies (MU-3 and 19B7), and urinary γ-carboxyglutamic acid (γ-Gla) in healthy adults (45 males and 45 females) and 11 patients with liver cirrhosis. VK and γ-Gla were analyzed by reverse-phase HPLC, prothrombin was determined using a method with a synthetic chromogenic substrate, and DCPs were analyzed by immunoaffinity chromatography and reverse-phase HPLC, and measured by ELISA. We were able to detect phylloquinone K (PK) and menaquinones MK-4, -5, -6, -7, -8 and -9, and we isolated several DCPs from plasma. Age had significant positive correlations with levels of urinary γ-Gla, plasma MK-7 and total VK, and VKDPs (prothrombin and DCP using MU-3 antibody) in the healthy adults. Significant positive correlations were also found between urinary γ-Gla and plasma levels of PK, MK-4, MK-7, and total VK and VKDPs (prothrombin and DCP using MU-3 antibody) in the healthy adults. γ-Gla and total VK levels were significantly higher in healthy adults than in patients with liver cirrhosis, and VK components, prothrombin, DCPs, and the MU-3/19B7 ratio showed a tendency to be higher in the healthy adults. We conclude that the status of VK and related proteins may reflect the latent increase in des-γ-carboxylation in healthy aging.

Keywords: Vitamin K (VK); Γ-Carboxyglutamic Acid (Γ-Gla); Prothrombin; Aging; Des Γ-Carboxyprothrombin (DCP)

1. Introduction

Vitamin K (VK) has two main forms, phylloquinone K (PK; VK_1) and menaquinones (MK, VK_2), and is increasingly understood to have important biological roles. MKs contain an unsaturated aliphatic side chain with a variable number of prenyl units; therefore, MKs are divided into short-chain (MK-4) and long chain (MK-5 to -13) subtypes. Both PK and MKs are cofactors for γ-glutamylcarboxylase, which catalyzes posttranslational conversion of specific glutamyl residues to γ-carboxyglutamyl residues in vitamin K-dependent proteins (VKDPs) involved in blood coagulation, bone and cartilage metabolism, signal transduction, and cell proliferation.

VK serves as a cofactor for γ-glutamylcarboxylase, which catalyzes conversion of a Glu residue of VKDPs into γ-carboxyglutamic acid (Gla). This process is driven by oxidation of VKH_2 (hydroquinone) to VKO (epoxide) in the VK cycle. Since γ-Gla in a Gla-containing protein is stoichiometrically excreted into urine as free Gla, urinary Gla excretion is believed to reflect the rate of synthesis and degradation of VKDPs and utilization of VK *in vivo*.

VK is an essential nutrient that must be obtained from the diet or dietary supplements. The primary dietary form of VK is PK, which is found in green leafy vegetables and vegetable oils. MKs are found in animal-based and fermented foods or produced by bacteria in the human gut. PK and MKs are absorbed (together with dietary fat) from the small intestine and transported in the circulation by lipoproteins [1, 2].

* Corresponding author: Soichiro Uehara
Pharmacy Department, Hijirigaoka Hospital, Department of Gastroenteronology Hijirigaoka Hospital Hokkaido, Japan.

Recently, MK-4 has been noted as an exception because it is not a common product of bacterial synthesis, but is considered to be of animal origin based on its tissue-specific conversion from PK. Menadione, although often referred to as vitamin K3, is not a natural component of foods, but is a product of catabolism of PK and a circulating precursor of tissue MK-4. Regarding the bioavailability and biodistribution, MKs have a longer half -life in the circulation than PK. PK is retained and exerts its function in the liver, whereas MKs are redistributed to the circulation and extrahepatic tissues [3].

In the past two decades, the health benefits of VK have been shown to extend beyond coagulation, and VK is now known to have a role in ensuring the correct function of several extrahepatic VKDPs that act in physiological and pathological processes, such as tissue mineralization, neuroprotein, energy metabolism, inflammation and cellular growth and survival [1,2].

Recently, the use of VK in prevention and clinical treatment of chronic age-related diseases (cardiovascular diseases, osteoporosis and osteoarthritis), inflammation-related diseases, neurological disorders and cancer has also been discussed.

In this study, we first examined the plasma VK levels in a group of healthy adults to assess differences due to age and sex, and we added a group of patients with liver cirrhosis with VK-deficiency [4, 5].

Des-γ-carboxylate prothrombin (DCP) is measurable in the circulation, and DCP concentrations change in response to dietary VK deficiency, warfarin use, and liver diseases such as hepatocellular carcinoma (HCC) and liver cirrhosis. We purified DCPs from the plasma of patients and healthy adults and analyzed the Gla content and heterogeneity by reverse HPLC. Subsequently, we measured the levels of 1-, 3- and 4-Gla DCPs by ELISA using monoclonal MU-3 antibody and 6-, 7- and 8-Gla DCPs using 19B7 antibody [6, 7].

The levels of urinary γ-Gla and plasma prothrombin were also measured. Correlations with age, sex and γ-Gla were examined to evaluate the significance of the VK status in healthy adults.

2. Methods

2.1. Subjects

The study was approved by the ethics committee of Hijirigaoka Hospital and complies with the Treaty of Helsinki. Eleven patients with liver cirrhosis and 90 healthy volunteers were included in the study. All patients and volunteers gave informed consent and all agreed to provide blood samples, urine and clinical information for the study. The 90 healthy adults (45 males and 45 females, mean BMI 24.6 kg/m^2) were aged 20 to 84 years.

The 11 patients with liver cirrhosis with suspected VK deficiency were added for comparison. The daily diet for a week prior to blood and urine collection included 300 g of carbohydrate, 40-50 g of fat, 70-80 g of protein (ca. 2000 Kcal/day). Consumption of Natto was prohibited for one week before blood sampling. The healthy adults had all liver and kidney functions and levels of red blood cells, white blood cells, platelets, cholesterol and triglyceride in the normal range.

2.2. Measurement of vitamin K levels

VK (PK and MKs) were measured with the method described by Shino, using a platinum oxide catalyst reduction column (Kawaken Fine Chemicals Ltd., Tokyo, Japan) [8].

In brief, 5 ng of MK-3 (Eizai Co., Ltd., Tokyo, Japan) as the internal standard was added to a 1-ml blood sample from each subject, and the sample was extracted with hexane and ether, and then with hexane alone. The sample was then refined on a silica gel column (Wako Pure Chemicals Ltd.) before injection into a HPLC apparatus for measurement. Quantification was performed using a standard curve determined with various concentrations of PK and MK-4 to -9.

2.3. Measurement of γ-Gla levels

γ-Gla was measured by the method of Kuwada and Katayama using o-phthalaldehyde. The supernatant of urine obtained after centrifugation at 3000 rpm for 10 min was diluted and subjected to HPLC. The urinary creatinine concentration was measured at the same time [9].

2.4. Measurement of plasma prothrombin

Plasma prothrombin was measured using the method of Kornalik et al. with a synthetic chromogenic substrate (S-2238; Kabi Diagnostica, Stockholm, Sweden) [10].

2.5. Isolation of prothrombin

Plasma samples were expanded in PBS buffer (pH 7.4) and added to tosyl-activated magnetic beads (Dynabeads-280; Dynal, Oslo, Norway) coated with polyclonal anti-human prothrombin antibody (Dako, Glostrup, Denmark). After incubation, the beads were removed from the mixture and harvested prothrombin was condensed and desalted. Isolated prothrombin was analyzed by reverse-phase HPLC in a column (Phenyl 5PWRP, Tosoh, Tokyo, Japan) using solution A containing H_2O with 0.1% trifluoroacetic acid (Kishida Chemicals, Osaka, Japan) with 0.1% TFA. The ratio of solution A to B was 9:1 from 0-2 min, and 3:7 from 2.1-90 min. The flow rate was 1 ml/min and the detection wavelength was 280 nm (7). Total DCP was obtained by summing the peak areas of each DCP variant on HPLC analysis. The level of each variant was expressed as a percentage of total DCP.

2.6. Measurement of des-γ-carboxyprothrombin (DCP) using MU-3 and 19B7 antibodies

Plasma DCP was measured using an ELISA kit (Eitest PIVKA-II kit; Eizai, Tokyo, Japan) with MU-3 and 19B7 antibodies. The MU-3/19B7 ratio was calculated [6].

2.7. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was carried out by Student t-test and the level of significance was $P< 0.05$.

3. Results

3.1. Plasma levels of vitamin K and γ-Gla

PK and MK-4, -5, -6, -7, -8, and -9 levels in plasma were measurable using our method. These levels and those of total VK and urinary γ-Gla in healthy adults and patients with liver cirrhosis are shown for each sex in Figure 1.

The plasma levels of PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, and total VK were (mean ± SD) 1.03±2.11, 0.89±0.74, 0.47±0.29, 0.30±0.22, 1.39±1.55, 0.23±0.35, 0.22±0.29, and 4.45±3.32 ng/mL, respectively, in healthy adults; and those of PK, MK-4, MK-7, MK-9 and total VK were 2.62±3.60, 1.35±2.04, 2.10±3.61, 0.22±0.09, 0.47±0.49, and 5.82±4.60 ng/mL, respectively, in patients with liver cirrhosis. All levels were higher in the patients, but with no significant difference. The levels were also higher in males, but again the difference was not significant.

The levels of urinary γ-Gla, plasma prothrombin, DCP by MU3 antibody, DCP by 19B7 antibody, and the MU-3/19B7 ratio were 79.2±13.5 nm/mg·Cr, 82.0±11.3 μg/ml, 21.9±9.0 mAU/ml, 16.8±5.4 mAU/ml, and 1.4±0.4, respectively, in healthy adults; and 60.9±19.9 nm/mg/Cr, 39.0±14.0 μg/ml, 50.0±63.5 mAU/ml, 29.0±547.3 mAU/ml, and 2.0±1.9, respectively, in patients with liver cirrhosis.

Urinary γ-Gla and plasma prothrombin were significantly lower in the patients, and DCP and the MU-3/19B7 ratio tended to be higher in the patients, but the difference was not significant (Figure 1).

3.2. Correlation between age and VK status

The average age of the healthy adults was 48.7±18.2 years and that of the patients with liver cirrhosis was 53.9±9.5 years. PK and MK-4 levels gradually increased with age (Figure 2), but the correlations of age with PK and MK-4 were not significant. There were weak significant positive correlations of age with plasma MK -7 and total VK (Figure 2).

We were unable able to detect MK-5, MK-6, MK-8, and MK-9 in most cases, and correlations with age were uncertain. The correlation coefficients of the total amount of MK-5, -6, -8 and -9 were -0.123 with age and -0.08 with γ-Gla. These values were not significant, but may show a tendency for a decrease with age. Overall, these results show that VK increases with age in healthy adults, which suggests that VK is required for normal physiological aging.

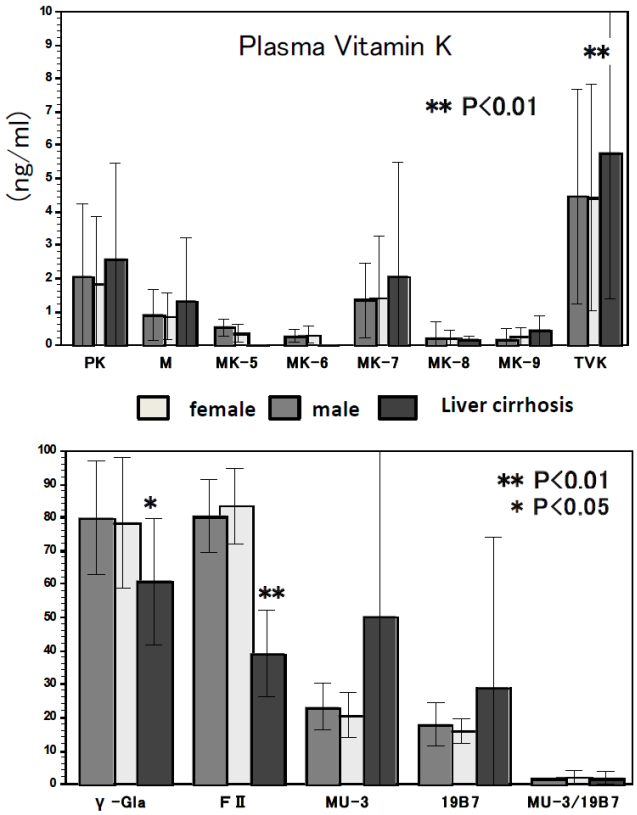

Figure 1 Distribution of plasma VKs (PK, MK-4,5,6,7,8,and -9, and total VK), prothrombin, DCP using MU-3 and 19B7 antibodies, MU-3/19B7 ratio of DCP, and urinary γ-Gla levels in healthy adults and patients with liver cirrhosis.

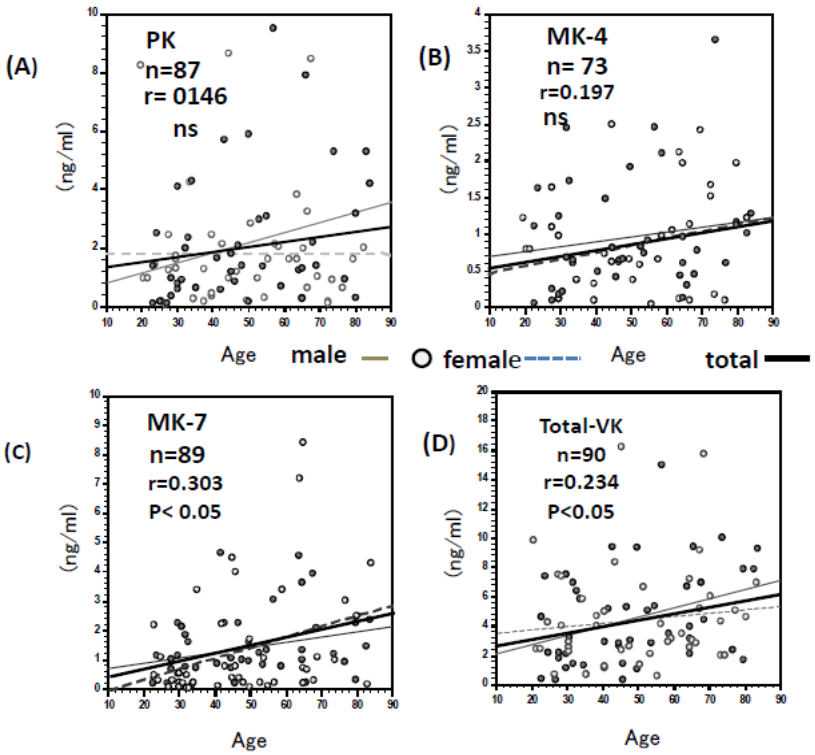

Figure 2 Relationships between age and levels of VKs [PK (A),MK-4(B),MK-7(C), and total VK(D)] in healthy adults (●male—, and ○female···,total VK—).

3.3. Relationship between plasma VK and urinary γ-Gla

There were weak but significant positive correlations between urinary γ-Gla and plasma PK, MK-4, and MK-7 in healthy adults (Figure 3), indicating a significant positive correlation between total VK and urinary γ-Gla. There was also a significant positive relationship between age and urinary γ-Gla levels in healthy adults.

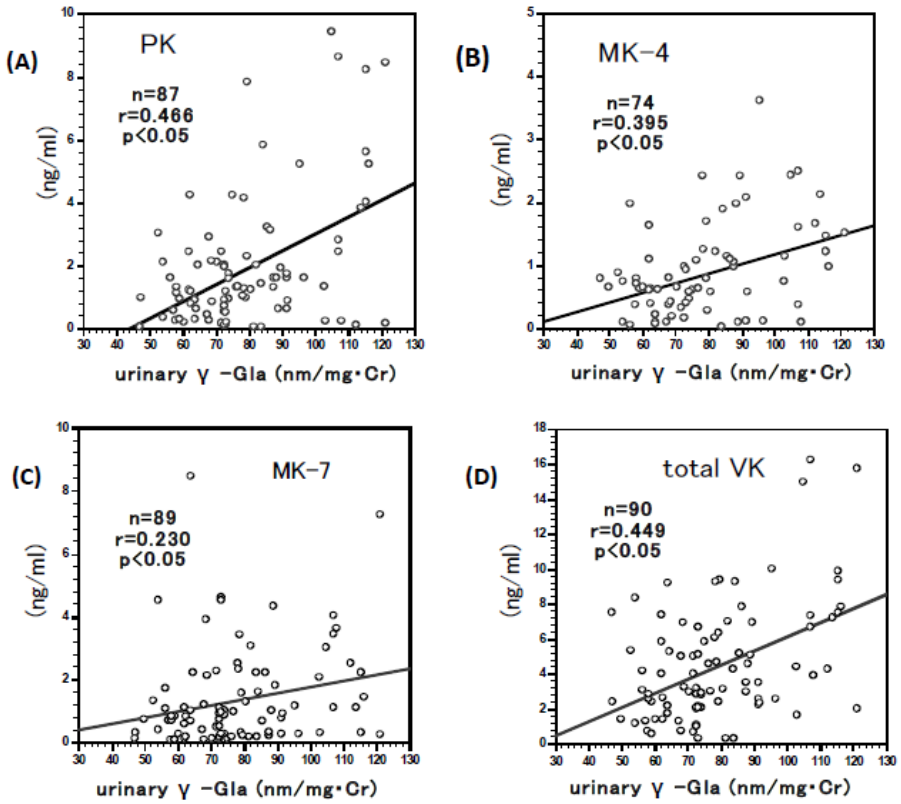

Figure 3 Relationships between levels of urinary γ-Gla and plasma VKs [PK(A), MK-4 (B), MK-7(C), and total VK(D)] in healthy adults.

3.4. Distribution of isolated DCPs variants

Isolated DCP variants from healthy adults and patients with liver cirrhosis are shown in Figure 4. The amount of the 10-Gla variant (mature prothrombin) was highest, and 3-, 6- and 5-Gla variants were found in healthy adults. A decrease of 10-Gla and increases of other DCP variants were found in the liver cirrhosis cases.

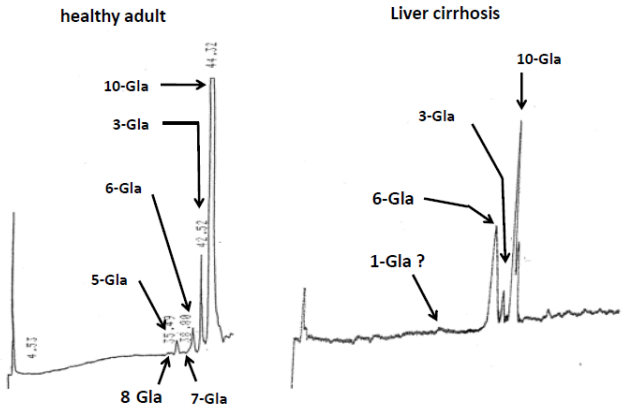

Figure 4 Profile of the DCPs was isolated from plasma of a healthy adult (A) and a patient with liver cirrhosis (B).

3.5. Plasma levels of prothrombin and DCP variants

The plasma levels of prothrombin, DCPs (using MU-3 and 19B7 antibodies) and the MU-3/19B7 ratio for DCPs are shown in Figures 1 and 5.

Plasma levels of prothrombin and DCPs using the two antibodies showed no apparent sex differences in healthy adults. The prothrombin level was significantly lower and DCPs were higher (but without significance) in liver cirrhosis cases compared to those in healthy adults. Plasma prothrombin did not increase with aging in healthy adults, and was approximately constant regardless of age.

DCPs in the healthy adults and patients with liver cirrhosis are shown in Figure 1, and the associated relationships are shown in Figure 3. DCP levels detected with MU-3 antibody were higher than those detected with 19B7 antibody in both groups, but the differences were not significant. The MU-3/19B7 ratio was higher in liver cirrhosis cases than in healthy adults, but also without a significant difference. Age was significantly positively correlated with the MU-3/19B7 ratio in healthy adults (Figure 5).

The level of plasma prothrombin and of DCPs using both antibodies showed significant positive correlations.(MU-3, r=0.235; 19B7, r=0.221, both P<0.05). Age was significantly positively correlated with the DCP level using MU-3 antibody, but not with the DCP level using 19B7 antibody (Figure 5).

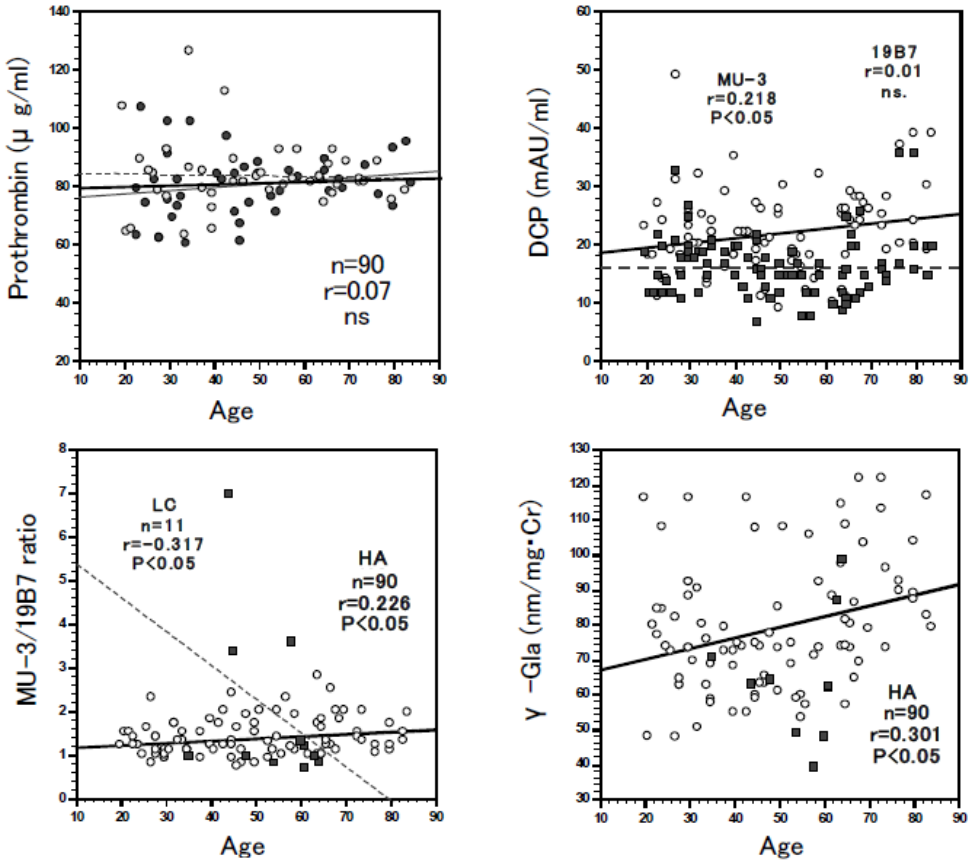

Figure 5 Relationships between age and levels of the plasma prothrombin (A), urinary γ-Gla (B), two DCPs (C), and MU-3/19B7 ratio (D) in healthy adults (A; • male, ○ female, B; •19B7, ○ MU-3, C; ▪ liver cirrhosis: LC, ○ healthy adult:HA, D; ▪ liver cirrhosis:LC).

The plasma prothrombin level and urinary γ-Gla levels were correlated in healthy adults and patients with liver cirrhosis (Figure 6). These correlations may distinguish healthy adults from patients with liver cirrhosis with VK deficiency.

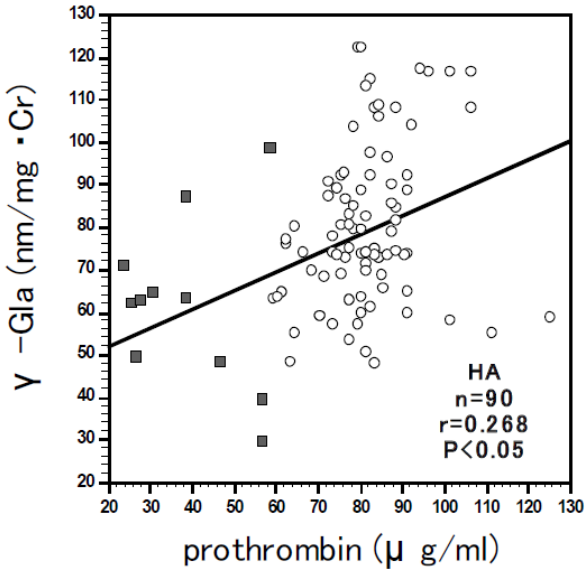

Figure 6 Relationships between plasma prothrombin levels and urinary γ-Gla levels in healthy adults and patients with liver cirrhosis (○ healthy control:HC, ▪ liver cirrhosis).

4. Discussion

VK was first reported as a cofactor for the microsomal enzyme γ-glutamylcarboxylase (GGCX) in ensuring the correct function of VK-dependent hepatic clotting factors. The posttranslational protein modification that converts specific glutamic acid residues (Glu) into γ-Gla is crucial for function of VKDPs, which are also known as Gla proteins. Current studies show that the health benefits of VK extend beyond coagulation, and VK roles include ensuring the correct function of several extrahepatic VKDPs. Roles in physiological and pathological processes such as tissue mineralization, neuroprotection, energy metabolism, inflammation, and cellular growth and survival are also now known, and use of MKs in prevention and treatment of cancer has been proposed [11]. Since γ-Gla in Gla-containing proteins is stoichiometrically excreted into urine as free Gla, urinary Gla excretion may reflect the synthesis and degradation rates of VKDPs and utilization of VK in vivo [12].

Development of a practical and high-throughput analytical method for VK homolog quantification is required due to increased interest in determination of VK concentrations in clinical and nutritional studies to better understand the role of VK in health and diseases. HPLC was developed for this purpose in the second half of the last century and extended to measurement of long-chain MKs in the late 1980s. HPLC is the elective technique to measure VK subtypes, but in the last 20 years, several detection methods have been reported for determination of VK in biological samples, including fluorescence detection (FD), ultraviolet detection (UV), electrochemical detection (ECD), photodiode array (PDA), chemiluminescence (CL), and mass spectrometric detection (MS or MS/MS).

The use of LC-MS/MS bioanalytical methods to determine trace levels of vitamin K in biofluids has markedly increased in the last decade because of its clear advantages of improved sensitivity, specificity and throughput. MS detection overcomes the need for a complex sample preparation procedure, but has its own limitations, including relatively expensive equipment and possible matrix effects causing interference in biological samples [13-15].

UV detection is cost-effective, but has been largely replaced by LC-MS/MS, which provides higher sensitivity and selectivity in comparison with other techniques, which is important for high analyte concentrations in limited sources of biological samples. However, measurement of plasma VK with reverse HPLC is performed widely. Therefore, our method using HPLC was chosen for its cost-effectiveness, speed, and relative accuracy.

Recent reports have described limits of detection for MK-4 of 0.04 ng/mL, PK of 0.03 ng/mL, and MK-7 of 0.03 ng/mL. MK-7 is commonly >5 ng/ml in vivo, and MK-4, PK and MK-7 have been found to be 0.15±0.17, 1.81±1.10 and 16.27±20.58 ng/mL, respectively, in healthy adults; and 46.83±46.41, 0.62±0.25 and 4.18±6.28, respectively, in patients with osteoporosis receiving MK-4 [16]. In another study, MK-4, PK and MK-7 were found to be 0.39±0.46, 1.22±0.57 and 6.37±7.45 ng/mL, respectively [13].

Our VK measurements did not show major differences to these reports. We also note that there are inconsistencies in reports of MK levels in different diseases. Collectively, these reports suggest a reference range for PK of 0.1-3 ng/mL. However, the reference ranges for MKs are still unclear, and there is currently no established threshold of MKs that indicates insufficiency or deficiency.

Our measured plasma levels of MK-4, -5, -6, -7, -8, -9 and PK are similar to those in previous reports. PK had the highest level, followed by MK-7 and MK-4.

The levels of MK-7 in our subjects may also be lowered by the influence of Natto. MK-7 is absorbed efficiently and has high bioavailability, which explains why postprandial serum levels of MK-7 are 10-fold higher than those of PK [17, 18].

PK also shows inferior absorption compared to MK-4, as well as longer-chain MK food sources (MK-8 and MK-9). Long-chain MKs that were detected sporadically, such as MK-7 and MK-9, have longer half-lives in circulation compared to PK due to their high lipophilicity, which causes these compounds to be poorly absorbed in extrahepatic tissues.

These findings suggest that PK is retained in the liver and exerts its function, whereas MK-n is redistributed to the circulation and extrahepatic tissues [14-18].

However, except for MK-7, there are few reports on the levels of the MK-5, -6, -8 and -9. Our results suggest that the levels of long-chain MKs may be high in younger people. Recently, Nakagawa et al. identified UbiA prenyltransferase containing 1 (UBIAD1) as the enzyme responsible for conversion of PK to MK-4 in several tissues.

The discovery of a MK-4 biosynthetic enzyme in humans and confirmation that MK-4 originates from PK might support the rationale of a lower efficacy of MK-4 compared to other MKs. Our results showed that VK levels in patients with liver cirrhosis with VK deficiency are still higher than in healthy adults [3].

Studies of VK in healthy adults must consider eating habits, and published data differ significantly among countries. MK-7 and total VK significantly increased with aging, and PK and MK-4 showed a tendency to increase. Thus, total plasma VK and urinary γ-Gla increase with aging in healthy adults. However, it is not currently known if the recommended intake is sufficient to meet all physiological needs. VK in males was only slightly higher than in females in healthy adults. Thus, there appears to be no significant sex difference in VK bioavailability, but age may be a nondietary factor influencing response. Thus, daily VK intake in healthy adults may be important [18,19].

It is difficult to examine clinical outcomes in patients to compare the effects of VK levels, and measurement of VKDPs is important. The main role of VK is as a cofactor for γ-glutamylcarboxylation of hepatic and extrahepatic proteins.

Plasma prothrombin and urinary γ-Gla levels were lower in patients with hepatic cirrhosis than in healthy adults. VK deficiency reduces the activity of VKDPs, which reduces the levels of dp-ucMGP, ucOC, and DCP, which are proteins that have a role in maintaining vascular, bone and hepatic health [20-22].

If γ-carboxylation of prothrombin does not occur, DCPs that lack the calcium-binding properties required for coagulation are generated. Monoclonal MU-3 antibody detects the 1-, 3-, and 4-Gla DCP variants, while the 19B7 antibody recognizes the 6-,7-, and 8-Gla DCP variants. Thus, for DCPs, the MU-3 antibody does not recognize the γ-carboxylation, in contrast to the 19B7 antibody [7].

The results of this study show that DCP increases with aging, and γ-Gla and VK components increase in parallel. However, plasma prothrombin does not increase with aging. The VK changes in healthy adults with aging were higher than sex differences. Viewing these results in an integrated manner, physical homeostasis is maintained with increased γ-carboxylation accompanied with increased des-γ-carboxylation in aging. This increase may extend beyond the liver to extrahepatic tissues. From recent studies, the role of VK in coagulation is well established, and a role in disease is recognized in cardiovascular disease, bone development and fractures, chronic kidney disease, and certain cancers. Additional evidence is indicating a further role of VK in liver disease, immune function, neurological disease and obesity [23].

Another study of VK levels in healthy adults is required to examine dietary habits in each disease. Accompanying clinical studies may require a long time, but should be continued to address the many areas of health that depend on VK.

5. Conclusion

DCPs with des-γ-carboxylation increase with age. Therefore, VK status in healthy adults may become a latent VK-deficient state with aging.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

Our study was approved by the ethics committee of Hijirigaoka Hospital and complies with the Treaty of Helsinki. Eleven patients with liver cirrhosis and 90 healthy volunteers were included in our study. All patients and volunteers give informed consent and all agreed, to prove blood samples, urine and clinical information for the study.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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