Effects of Vitamin K2 (Menatetrenone) on Atherosclerosis and Blood Coagulation in Hypercholesterolemic Rabbits

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ABSTRACT—γ-Carboxyglutamic acid (Gla)-containing protein, synthesized in the presence of vitamin K, has been found in atherogenic plaques, but the pharmacological effect of vitamin K on atherosclerosis is unclear. We examined whether vitamin K2 (menatetrenone) could affect the progression of both atherosclerosis and hypercoagulability in hypercholesterolemic rabbits. Vitamin K2 in daily doses of 1, 10 and 100 mg/kg was given with a 0.5% cholesterol diet for 10 weeks to 8 rabbits each. The plasma levels of total-cholesterol in the vitamin K2-treated groups were clearly lower than that of the hypercholesterolemic control group. The excessive dose of vitamin K2, even at the high dose of 100 mg/kg/day for 10 weeks, did not accelerate the progression of atherosclerosis and did not promote the coagulative tendency in the rabbits. In contrast, the vitamin K2 treatment (1 to 10 mg/kg/day) suppressed the progression of atherosclerotic plaques, intima-thickening and pulmonary atherosclerosis, the increase of ester-cholesterol deposition in the aorta, and both the elevation in plasma factor X level and increase in Hepaplastin® test value in the rabbits. These results indicate that the pharmacological dose of vitamin K2 prevents both the progression of atherosclerosis and the coagulative tendency by reducing the total-cholesterol, lipid peroxidation and factor X activity in plasma, and the ester-cholesterol deposition in the aorta in hypercholesterolemic rabbits.

Keywords: Vitamin K2 (menatetrenone), Hypercholesterolemia, Atherosclerosis, Blood coagulation, Factor X

It has been reported that vitamin K is essential for the γ-carboxylation of glutamic acid (Gla) residues within different protein classes such as vitamin K-dependent clotting factors and bone Gla-protein (1, 2). Vitamin K2 (menatetrenone, VK2) is clinically used over long periods as an important drug for blood coagulation. It was recently clarified that VK2 prevented bone loss in some animal models (3–5) and in a clinical double-blind study (6); VK2 has therefore been used as therapy for osteoporosis in Japan.

A plaque Gla-protein, atherocalcin, was recently isolated from hardened atherosclerotic plaques (7, 8), but the function of this Gla-protein has remained obscure. Levy et al. (7, 8) reported that the Gla-protein may be related to atherosclerotic calcification, but Van Haarlem et al. (9) and Gijsbers et al. (10) suggested that the Gla-protein might prevent calcium deposition. This raises the question of whether vitamin K could affect the progression of atherosclerosis in atherosclerotic animals.

In hypercholesterolemic rabbits, the activities of vitamin K-dependent clotting factors in plasma were shown to be higher than those in normolipidemic rabbits (11, 12), and the procoagulant activity was increased on the aortic endothelium (13), suggesting increased plasma coagulability. We investigated whether VK2 accelerates or suppresses the progression of atherosclerosis and hypercoagulability in hypercholesterolemic rabbits.

MATERIALS AND METHODS

Materials
Menatetrenone (VK2), 2-methyl-3-all trans-tetraprenyl-1,4-naphthoquinone, a vitamin K2 homologue, used in this study was chemically synthesized by Eisai Co. (Tokyo). The purity and quality were confirmed by high-performance liquid chromatography (HPLC); the purity (lot 84062101) was 100%. Cholesterol was purchased from Riken Vitamin Co. (Tokyo), coconut oil from Bousou Yushi Co. (Ibaraki), EDTA-2K from Nacalai Tesque Co. (Kyoto), and sodium citrate from Tokyo
Animals and their treatments

Sixty male four-month-old New Zealand white rabbits, weighing 2.6–2.9 kg, were purchased from Nihon SLC Co. (Shizuoka). The animals were housed individually in wired-bottomed aluminum cages in a room maintained at 22 ± 1 °C, with a 12-hr light-dark cycle. A regular diet (100 g/day) (RM-4; Funabashi Farm Co., Funabashi) and sterilized water ad libitum were given for three weeks. Then an atherogenic diet (100 g/day) consisting of the regular diet to which was added 0.5% cholesterol and 3% coconut oil was given to all rabbits for three days. On the third day, fasting blood samples were obtained from an ear vein, and plasma total-cholesterol levels were measured, and forty animals within the level of 200–400 mg/dl were selected. The animals were divided into 5 groups of 8 animals each and fed the regular diet for two weeks until their plasma total-cholesterol levels returned to the normal range. The control group was fed the atherogenic diet. The VK2 1 mg/kg/day group, the 10 mg/kg/day group and the 100 mg/kg/day group were fed the atherogenic diet supplemented with 0.003%, 0.03% and 0.3% VK2, respectively. The normal group was fed the regular diet. Approximately 100 g per day of the reconstituted pellets was given in the morning to each of the animals for 10 weeks, and the animals were weighed once each month. Sixteen-hour fasting blood samples (3 ml) were drawn from the vein with a needle into a plastic syringe containing 1% (v/v) of 40% EDTA-2K for lipids analysis, and blood samples (1 ml) containing 1% (v/v) of 40% EDTA-2K for lipids analysis, and blood samples (1 ml) containing 1% (v/v) of 40% EDTA-2K for lipids analysis, and blood samples (1 ml) containing 3.18% sodium citrate were used for blood coagulation analysis (Hepaplastin® test) at 0, 1, 2, 4, 6, 8 and 10 weeks. The blood was centrifuged at 4 °C at 3,000 rpm for 15 min, and the plasma was removed with a plastic pipette.

Lipid analysis

The plasma total-cholesterol level was measured enzymatically by an assay kit (Nescauto® TC-V5; Nippon Shoji Kaisha, Osaka). Plasma triglyceride and HDL-cholesterol levels were measured with the Triglyceride-S-R1® kit (International Reagents Co., Kobe) and HDL-C2-Daichi® kit (Daichi Pure Chemicals Co., Tokyo). The extent of plasma peroxidized lipids was measured in terms of thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde equivalents by the Yagi method (14) (Peroxidized Lipids® Test; Wako Pure Chemical Ind., Osaka). Aortic total-cholesterol and free-cholesterol contents were measured enzymatically with Determiner®-TC555 and Determiner®-FC555 kits (Kyowa Medics, Tokyo), respectively.

Coagulation assays

The blood coagulation characteristic was mainly measured with a Hepaplastin® test kit (Eisai Co.) known to be sensitive for vitamin K-dependent coagulation factors (15). Other coagulation assays were carried out only at 10 weeks. Plasma fibrinogen level was measured using Data-Fil® fibrinogen (American Dade, Aguade, Puerto Rico). One portion was frozen at −20 °C for the analyses of prothrombin (F-II), factors VII and X using Chromo-quick® (Behring-werke AG, Marburg, Germany) (16). These results are expressed as the average percentage change (±S.E.M.) of activity in each group as compared with that in standard rabbit plasma.

Plasma VK2 analysis

A blood sample (1.5 ml) was taken with a needle into a plastic syringe containing 1% (v/v) of 40% EDTA-2K after 5 hr of feeding, at 3 and 7 weeks. Plasma was stored at −20 °C until analysis. The plasma VK2 concentration was determined by HPLC following extraction with n-hexane/isopropanol/water (10 : 3 : 1, v/v/v) (17).

Atherosclerosis determination

At the end of the 10-week experimental period, animals were sacrificed by exsanguination under pentobarbital anesthesia. The thoracic aorta was rapidly obtained and opened longitudinally and divided into four parts of two aortic arches and two descending aortae. One arch and one descending aorta were immediately frozen in liquid nitrogen and stored at −20 °C until analysis. The other two parts were fixed in 10% neutral-buffered formalin. The surface area of atheromatous lesions stained with Sudan IV was evaluated planimetrically from a photograph with an image analyzer (SPICCA-ACE Model-1500c; Nippon Avionics Co., Tokyo) and expressed as a percentage of total aortic intimal surface area. The aorta stained with Sudan IV was refixed in 10% neutral-buffered formalin, and paraffin-embedded sections were stained by the method of Masson & Goldner for measurement of the intimal and medial areas. The intimal thickness was estimated by the following formula: The thickness (%) = {intimal thickness ÷ (medial thickness + intimal thickness)} × 100.

Pulmonary atherosclerosis determination

The lung was removed with the aorta from rabbits at the same time and fixed in 10% neutral-buffered formalin. Paraffin-embedded sections of the lungs were stained with hematoxylin-eosin, and the photographic evaluation of pulmonary arteries in a visual field was based on visual analogue scores of one to five, in which one (−) is normal, two (+) is mild, three (++) is moderate, four (+++) is severe and five (++++) is very severe.
Aortic cholesterol determination

The aortic arch and descending aorta were homogenized with saline using a Physcotron® (Nich-on Mfg. Co., Chiba). Total and free cholesterol were extracted with chloroform/methanol (2:1, v/v) and then measured quantitatively.

Statistical analyses

The values represent the means±S.E.M., and statistical significance was tested by one-way or split-block analysis of variance (ANOVA) using Fisher’s least significant differences method where data were normally distributed. For non-parametric data, the precision accumulation analysis by Taguchi (18) was used to analyze differences in frequencies and proportions between groups. Significance was accepted at P values less than 0.05.

RESULTS

Body weight

All the rabbits survived to the end of the 10 weeks, and they gained weight during the 10 weeks of feeding. No significant difference was observed in the body weights among the 5 groups from the start to the end of the experiment. At the time of sacrifice, the body weights of the 40 animals were in the range of 3.1–3.9 kg.

Plasma total-cholesterol concentration

Plasma total-cholesterol levels in the atherogenic control group increased rapidly and reached maximum values at about 8 weeks, as shown in Fig. 1. In contrast, the levels in the VK2-treated groups were significantly lower than those in the atherogenic control group at 8–10 weeks. At the end of the experiment, the level in the atherogenic control group was 3238±379 mg/dl. The levels in the VK2-treated groups were 2730±244 mg/dl in the 1 mg/kg group, 2384±464 mg/dl in the 10 mg/kg group (P<0.05 vs control) and 2446±186 mg/dl in the 100 mg/kg group (P<0.05 vs control).

Plasma peroxidized lipids concentration

As shown in Fig. 2, a linear increase in the plasma peroxidized lipids concentrations was observed until 4 weeks, and the concentration was maintained at high levels in the control rabbits fed the high cholesterol diet. The peroxidized lipids level in the VK2 1 mg/kg group was significantly lower than that in the control group at 4 weeks. A significant decrease in the level in the VK2 10 mg/kg group was also observed at both 4 and 6 weeks, and the level in the VK2 100 mg/kg group was slightly lower than that in the control group.

Plasma triglyceride and HDL-cholesterol levels

The plasma triglyceride levels (Table 1) in the four groups of rabbits fed the atherogenic diet gradually increased until 10 weeks, and the HDL-cholesterol levels (data not shown) of these rabbits were gradually increased until 4 weeks and then decreased thereafter. There were no significant differences among the four groups in both plasma triglyceride and HDL-cholesterol levels.

Plasma VK2 concentration

Dose-dependent significant increases in the plasma VK2 concentrations were observed in the VK2-treated

![Fig. 1. Effect of VK2 on plasma total-cholesterol levels in hypercholesterolemic rabbits.](image)

![Fig. 2. Effect of VK2 on plasma peroxidized lipids concentration (TBARS) in hypercholesterolemic rabbits.](image)
groups at 3 and 7 weeks (Table 2). The level in the VK2 100 mg/kg group at 7 weeks, in particular, was slightly higher than that in the same group at 3 weeks.

**Table 2.** Plasma menatetrenone (VK2) concentration in hypercholesterolemic rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma VK2 concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.4±0.4</td>
</tr>
<tr>
<td>VK2 1 mg/kg</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>VK2 10 mg/kg</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>VK2 100 mg/kg</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.M. of 8 rabbits.

As shown in Fig. 3, marked increases of the ester-cholesterol contents both in the aortic arch and the thoracic aorta were observed in the cholesterol-fed control rabbits. In the VK2 100 mg/kg group, there was no significant difference in the ester-cholesterol contents. However, significant decreases of the ester-cholesterol contents in the aortic arch and the thoracic aorta were noted in both the VK2 1 mg/kg and 10 mg/kg groups.

**Aortic ester-cholesterol content**

As shown in Fig. 3, marked increases of the ester-cholesterol contents both in the aortic arch and the thoracic aorta were observed in the cholesterol-fed control rabbits. In the VK2 100 mg/kg group, there was no significant difference in the ester-cholesterol contents. However, significant decreases of the ester-cholesterol contents in the aortic arch and the thoracic aorta were noted in both the VK2 1 mg/kg and 10 mg/kg groups.

**Aortic atherosclerosis**

The endothelial surface of the aortic walls was examined for atherosclerotic plaques. In the control group rabbits, a marked development of atherosclerotic plaques had occurred, and the plaque formation was greater in the aortic arch than in the thoracic aorta. A significant decrease of the percentage of plaques in the thoracic aorta was observed in the VK2 1 mg/kg group, and the percentages of plaques were slightly decreased in the VK2 10 mg/kg group, both in the aortic arch and the thoracic aorta (Table 3), although there was no significant difference in the development of atherosclerotic plaques in the VK2 100 mg/kg group.

The histological sections through atherosclerotic plaques of the aortae showed the thickening of the intima in the rabbits fed the high cholesterol diet. The thickness of the intima in the VK2 1 mg/kg group was slightly...
decreased compared with that in the control group, and a significant decrease of the thickness was observed in the VK2 10 mg/kg group (Fig. 4), although there was no difference in the thickness of the intima between the VK2 100 mg/kg group and the control group.

### Pulmonary atherosclerosis

Severe pulmonary atherosclerosis was observed in the control rabbits. No significant differences in the degree of pulmonary atherosclerosis were found among the VK2 1 mg/kg, VK2 100 mg/kg and control groups. However, in the VK2 10 mg/kg group, the degree of pulmonary atherosclerosis was significantly lower than that in the control group (Fig. 5).

### Plasma Hepaplastin® test value

Plasma Hepaplastin® test value in the control group gradually increased, and the values at 4, 6, 8 and 10 weeks were significantly higher than that in the normal rabbits (Fig. 6). At the 4th week, the value in the VK2 1 mg/kg group was slightly lower than that in the control group (P < 0.10), and the value in the VK2 10 mg/kg group was significantly lower than that in the control group (P < 0.05). At the 10th week, the values in the VK2 1 mg/kg and 10 mg/kg groups were slightly lower than that in the control group.

### Plasma prothrombin and factor VII levels

Plasma prothrombin and factor VII levels were compared among the groups at the 10th week (Table 4). The plasma prothrombin level in the control group was significantly higher than that in the normal group, but there...
were no differences among the VK2-treated groups. There were no differences in the plasma factor VII levels between the atherogenic groups and the normal group.

**Plasma factor X level**

The factor X activity in the control group was 272±20% at the 10th week. The activities in the VK2-treated groups were significantly lower, with values of 176±8% in the 1 mg/kg group (P<0.01 vs control), 164±19% in the 10 mg/kg group (P<0.01 vs control) and 213±19% in the 100 mg/kg group (P<0.05 vs control), as shown in Fig. 7.

**Plasma fibrinogen level**

At the 10th week, the plasma fibrinogen level in the atherogenic control group was significantly lower than that of the normal rabbits, but no significant differences were observed between the VK2-groups and the control group (Table 4).

Table 4. Plasma prothrombin and factor VII activities (%) and fibrinogen levels in hypercholesterolemic rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma prothrombin (μ%)</th>
<th>Plasma factor VII (μ%)</th>
<th>Plasma fibrinogen (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100±6</td>
<td>100±7</td>
<td>195±9</td>
</tr>
<tr>
<td>Control</td>
<td>134±11</td>
<td>111±10</td>
<td>152±9</td>
</tr>
<tr>
<td>VK2 1 mg/kg</td>
<td>141±5</td>
<td>104±6</td>
<td>146±9</td>
</tr>
<tr>
<td>VK2 10 mg/kg</td>
<td>121±6</td>
<td>102±6</td>
<td>166±16</td>
</tr>
<tr>
<td>VK2 100 mg/kg</td>
<td>142±8</td>
<td>123±8</td>
<td>153±8</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.M. of 8 rabbits. Significance level is denoted by: *P<0.05, **P<0.01.

DISCUSSION

It has been reported that vitamin K is essential for the γ-carboxylation of vitamin K-dependent blood clotting proteins (19, 20). Little is known, however, about the role of vitamin K in atherosclerosis (2). This is the first study to report the effect of VK2 on atherosclerosis and hypercoagulability in hypercholesterolemic rabbits. Although the lack of effect of VK2 excess on coagulability was reported in normal rabbits in a study from our laboratories (21), the effects of VK2 on both atherosclerosis and hypercoagulability have not been investigated in hypercholesterolemic animals.

In the present study, surprisingly, the deterioration of atherosclerosis was not produced by the administration of an excessive dose of VK2, but the pharmacological dose of VK2 significantly prevented the deposition of ester-cholesterol in the aorta of the hypercholesterolemic rabbits.

A significant prevention of the increase in plasma total-cholesterol was produced by VK2 at doses of 1 to 100 mg/kg/day in the rabbits (Fig. 1). The prospective Framingham study showed that a risk of coronary heart disease in persons older than 50 was strikingly related to the serum total-cholesterol level (22). The findings that the atherosclerotic plaques exhibited an increasing accumulation of lipids predominantly in the form of ester-cholesterol (23) and that diet-induced hypercholesterolemia produced intimal atherosclerotic lesions (24) also supported the contention that hypercholesterolemia is a major risk factor for atherosclerosis (22). Therefore, the suppression of the increase in plasma total-cholesterol by VK2 might have played the most important role in preventing the deposition of ester-cholesterol in the aorta of the hypercholesterolemic rabbits (Fig. 3).

The clinical dosage of VK2 generally used as treatment for osteoporosis is 15 × 3 mg/patient/day (i.e., 0.75 mg/kg/day); the 100 mg/kg/day administered in the present study is 133 times higher than the clinical dosage.
A dose 100 times higher than the pharmacological dose is commonly used for studying the toxicity of safe agents; the 100 mg/kg/day dose was therefore used as the high dose in this study.

The deterioration of atherosclerosis in the rabbit aorta and pulmonary arteries was not observed after the long-term administration of an excessive amount of VK$_2$, even at the high dose (100 mg/kg/day). In contrast, the suppression of atherosclerotic development in the aorta and pulmonary arteries was produced by the administration of VK$_2$. VK$_2$ at a dose of 1 mg/kg/day tended to prevent the progression of the thickening of the intima; it significantly suppressed the thoracic atherosclerotic plaques, and it significantly decreased the aortic ester-cholesterol content. At the dose of 10 mg/kg/day, a significant suppression of the progression of intima-thickening and pulmonary atherosclerosis, and a significant decrease of the aortic ester-cholesterol content were observed (Figs. 3-5, Table 3). Therefore, the pharmacologically effective dose of VK$_2$ for preventing the progression of atherosclerosis as determined histologically was concluded to be 1 to 10 mg/kg/day.

At the high dose (100 mg/kg/day), a significant decrease in the plasma total-cholesterol level was observed, but a histological suppression of atherosclerosis was not observed. The reason for this loss of suppression of atherosclerosis at the high dose of VK$_2$ is unclear, but possible mechanisms include a nonspecific toxic action of VK$_2$ on the cholesterol and/or calcium metabolisms in the aorta.

VK$_2$ at 1 and 10 mg/kg/day significantly suppressed plasma lipid peroxidation (Fig. 2). Vitamins K$_2$ and K$_1$, unlike vitamin K$_3$, inhibited the lipid peroxidation of lecithin liposomes induced by ascorbic acid and ferrous ions (25). Although the inhibitory mechanism of lipid peroxidation of VK$_2$ is unclear, Kruk et al. (26) reported that the reduced form of VK$_2$ showed a stronger free-radical-scavenging action in vitro than did α-tocopherol, known to inhibit the oxidative modification of low-density lipoproteins (LDL) (27) and to prevent the progression of atherosclerosis in hypercholesterolemic rabbits (28). These lines of evidence indicate that VK$_2$ may prevent the formation of plasma-peroxidized lipids and plasma-oxidized LDL, and thus prevent the progression of atherosclerosis in hypercholesterolemic rabbits.

It has been reported that the activities of vitamin K-dependent clotting factors in plasma were enhanced in hypercholesterolemic rabbits (11, 12), that oxidized LDL reduced the inhibitory effect of endothelium on platelet adhesion and aggregation (29), and that oxidized LDL increased the procoagulant capacity of the endothelium by enhancing the expression of tissue factor (30, 31). As shown in Table 4 and Figs. 6 and 7, significant increases both in the levels of plasma factors II and X and in the Hepaplastin$_{\text{B}}$ test value were observed in the hypercholesterolemic control rabbits. These high levels of plasma factors II and X may contribute to the progression of thromboatherosclerosis (30). It is therefore possible that an excess of vitamin K may promote the procoagulant activity in hypercholesterolemic and hypercoagulable rabbits. However, the long-term administration of an excessive amount of VK$_2$ even at a high dose of 100 mg/kg/day did not induce the acceleration of blood coagulation, but VK$_2$ significantly prevented the increase of plasma factor X activity in hypercholesterolemic and hypercoagulable rabbits (Fig. 7). The finding that the excessive dose of VK$_2$ did not accelerate the hypercoagulable state in hypercholesterolemic rabbits is in accord with our previous finding (21) that an excessive dose of VK$_2$ showed no effect on blood coagulation in normal rabbits. Vitamin K is not only essential for the formation of Glu-residues in several blood coagulation factors (II, VII, IX and X) but is also essential for the Gla-formation in negative coregulators in the blood coagulation cascade (proteins C and S) (32, 33). Thus, VK$_2$ may not always accelerate blood coagulation but may rather promote the synthesis of negative coregulators in the blood coagulation system, especially in the hypercoagulable state such as in hypercholesterolemic animals.

In this study, a significant decrease in plasma fibrinogen was observed at the 10th week in the hypercholesterolemic rabbits (Table 4). A similar finding was reported by Criscuoli et al. (34). In hypercholesterolemic rabbits, increased prothrombin and Factor X in plasma may lead to an increase in the production of thrombin and may subsequently contribute to the decrease of plasma fibrinogen levels.

It has been reported that atherosclerosis is a complex multifactorial process and that two key events are i) the deposition of ester-cholesterol and ii) the migration and proliferation of arterial smooth muscle cells (35). Corsini et al. (36) reported the specific role of isoprenoid metabolites, most probably geranylgeranylated protein(s) in the regulation of cell migration and proliferation. Since VK$_2$ has a geranylgeranyl residue in its molecule, it is possible that VK$_2$ is involved in the regulation of cell migration and proliferation.

A recent finding (37) that VK$_2$ inhibited vitamin D$_2$-induced experimental calcinosis of the aorta in rats suggests that VK$_2$ participates in the suppression of the deposition both of cholesterol and calcium in the aorta.

Finally, our results suggest that pharmacological doses of VK$_2$ play an important role in the prevention of atherosclerosis by reducing the total-cholesterol and lipid peroxidation in plasma and the ester-cholesterol deposition in the aorta in hypercholesterolemic rabbits.
Acknowledgments

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