〈Original Article〉

The effect of inhibition of vitamin K dependent coagulation factors on the progression of atherosclerosis in apoE$^{-/-}$LDLR$^{-/-}$ double-knockout mice

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Summary *Background*- Atherosclerosis is characterized by a hypercoagulable state indicating the relation with activated coagulation factors in the progression of atherosclerosis. The purpose of the present study was to investigate the contribution of vitamin K dependent coagulation factors to the progression of atherosclerosis in apoE$^{-/-}$LDLR$^{-/-}$double-knockout mice (DKO mice).
Methods and Results- We fed DKO mice a high-fat diet containing warfarin (INR: 1.98) 12 weeks. The amount of atherosclerosis was estimated as the ratio of atherosclerotic area, and nitric oxide releasing in vivo was measured by using electrochemical sensors. The expression of eNOS and LOX-1 in the atherosclerotic area was analyzed by immunohistochemistry. 8-OHdG was determined as a marker of oxidative stress. The progression of atherosclerosis was reduced significantly in warfarin group compared with placebo group.

The amount of nitric oxide release was increased in warfarin group compared with placebo group. In support of these results, immunohistochemistry showed increased eNOS expression in warfarin group. LOX-1 expression in placebo group was greater than in warfarin group and urinary 8-OHdG levels in warfarin group was lower than in placebo group.
Conclusion- Treatment with warfarin led to reducing atherosclerotic progression through reduction of oxidative stress and an increase of acetylcholine-induced NO release. These findings indicate that vitamin K dependent coagulation factors may be risk factors for the progression of atherosclerosis.

Key words: Thrombin, Nitric oxide, atherosclerosis, Warfarin, Vitamin K

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1. Introduction

Hypercholesterolemia, diabetes, and hypertension are prominent risk factors of atherosclerotic disease. High blood levels of low-density lipoprotein (LDL) promote the development of atherosclerosis, which often leads to ischemic heart disease and cerebral vascular disorders[1]. Several trials with statin have demonstrated decreased cardiovascular events and mortality due to lipid-lowering therapy[2-6].

However, in a large-scale clinical trial, a HMG-CoA reductase enzyme inhibitor decreased adverse events in only 20 % to 40 % of the population, with little effect in the majority of subjects[7,8]. This finding suggests that another factor in addition to dyslipidemia is involved in the progression of atherosclerosis. Increases in the activation of platelets and blood coagulation factors and in the metabolic turnover of fibrinogen are recognized to occur in some cases of atherosclerosis[9] and are potential risk factors for thrombotic complications.

Because atherosclerosis is characterized by a hypercoagulable state indicating the involvement of activated coagulation factors in the progression of atherosclerosis, anticoagulation could have beneficial effects on atherosclerosis. Coagulation factors can also affect the endothelial cell through regulation of the proliferation, migration and differentiation of vascular smooth muscle cells as well as by inducing oxidative stress, inflammation and apoptosis[10,11], all these processes that contribute to the progression of atherosclerosis.

Coagulation factors consists of several groups and vitamin-K dependent factors is the biggest group in them. Therefore, regulating vitamin K dependent factors leads to the effective control of coagulant activation. The vitamin K antagonist, warfarin, is the most commonly prescribed oral anticoagulant.

In the current study, we investigated the effect of oral administration of warfarin on the development of atherosclerosis and endothelial function in DKO mice.

2. Materials and methods

2.1. Experimental animals

Double-homozygous apoE-deficient, LDLR-deficient mice (apoE$^{-/-}$LDLR$^{-/-}$ DKO mice, 129 × C57BL/6J background, for warfarin group & placebo group) were obtained originally from the Jackson Laboratory (Bar Harbor, Maine, USA) and subsequently produced locally through sibling mating. C57BL/6 mice (male; age, 10–13 weeks, for control group) were obtained from SLC Co. Ltd. (Hamamatsu, Japan).

All mice were maintained at Kobe Gakuin University in air-conditioned rooms (22.5 ± 0.50 °C; humidity, 50 % ± 5 %) with a 12 h light-dark cycle. Only male mice were used in the present study. Animals had free access to diet and drinking water. Experimental diets were commenced from 6 weeks until 18 weeks of age.

All procedures were conducted in compliance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* of the Physiological Society of Japan.

2.2. The dose of warfarin

The dose of the warfarin (a generous gift from Eisai Co. Ltd, Tokyo, Japan) was decided referring to dose (1-5mg/day) based on the anticoagulant therapy in Japan. Three doses of warfarin were used at the pilot study.

During 12 weeks, amount of warfarin in feed is adjusted according to body weight and feed volume every week. The international normalized ratio (INR) values of PT in these dosage were 0.94 - 2.93. Finally, the warfarin group of INR value 1.98 was used for this study.

The dosage of warfarin in this study was selected as the highest concentration that did not cause the hemorrhagic complication by the pilot study based on a human anticoagulant therapy.

2.3. Experimental protocol

For 6 weeks prior to study initiation, both DKO and C57BL/6 male mice were fed a standard

commercial rodent chow (CE-2, CLEA Japan, Tokyo, Japan). C57BL/6 male mice was used as a blank group for immunohistochemistry, analysis of atherosclerotic blood vessel.

Warfarin group received an experimental diet, which was supplemented with warfarin. Placebo group fed experimental diet which was supplemented with non-functional agent (from Eisai Co. Ltd, Tokyo, Japan).

A control group of C57BL/6 mice was given the same feed as Placebo group. After the 12-week feeding period, atherosclerotic progression, endothelial cell function (in vivo NO release), organ pathology (immunohistochemistry and histology), and prothrombin time (PT), plasma nitrate and nitrite, cholesterol level and urinary 8-OHdG concentrations were evaluated.

2.4. Preparation of experimental diets

Experimental diets were prepared from a common standardized feed (AIN93G) by using the following purified materials: milk casein, f3 corn starch, sucrose, cellulose powder, mineral mixture, vitamin mixture (Oriental Yeast Co. Ltd., Tokyo, Japan), L-cystine, cholesterol (Nacalai Tesque Inc., Kyoto, Japan), choline bitartrate, and tert-butylhydroquinone (Wako Pure Chemical Industries Co. Ltd., Tokyo, Japan).

The composition of the experimental diet was 40 % lipid, 40 % carbohydrate, and 20 % protein. To this standardized feed, cholesterol was added at 0.05 % (w/w); warfarin (652 μg-2.6 mg/kg food) was added to individual aliquots of the experimental diet. Dose per day was calculated from the mean body weight (approximately 22- 40 g) at each weeks (6-18 weeks). The experimental food has been adjusted from mouse's food intake of a day. The powdered diets were solidified by adding agar (0.6 %) and were stored at –30 °C until use.

2.5. Measurement of atherosclerosis

The progression of atherosclerosis was assessed by estimating the area of atherosclerotic regions as a percentage of the entire surface area of the aorta, as previously described[12].

Briefly, hearts were exposed by abdominal incision, and phosphate-buffered saline (PBS; pH 7.4) was infused through an indwelling 20-gauge butterfly needle (Top Kasei Co. Ltd., Tokyo, Japan) followed by 10 % neutral-buffered formalin (Nacalai Tesque Inc.). In addition, the major blood vessels were washed with PBS and fixed with 10 % neutral buffered formalin solution by reflux through a femoral artery. Connective tissue and minor branching blood vessels were carefully removed from the aortic arch.

The extracted vessels were kept in 10 % neutral buffered formalin solution until processed, when they were incised along the longitudinal plane and pinned flat. The tissue was washed with distilled water for 30 seconds, treated with 60 % isopropyl alcohol for 1 minute, and stained with oil red O (to identify atherosclerotic plaques) at 37 °C for 15 minutes. Finally, the tissue was washed with 60 % isopropyl alcohol and distilled water.

2.6. Image analysis of atherosclerotic blood vessels

The stained specimens were photographed (PENTAX K7, Ricoh Company Co. Ltd., Tokyo, Japan); images were transferred to a personal computer and analyzed using image analysis software (Image-Pro Plus, Media Cybernetics, Rockville, Maryland, USA).

The whole area (W) of the dissected blood vessel and the portion that stained positively with oil red O (R) were calculated; the ratio [(R ÷ W) × 100 %] was used as an index of atherosclerotic progression.

2.7. Measurement of in vivo NO release

Peripheral NO release was measured by using electrochemical sensors as previously described[13,14].

Briefly, the working electrodes were introduced, without impeding blood flow, through a femoral arteriole and positioned near the branch point of the abdominal aorta and the common iliac artery. The electrodes were calibrated before each use by using a graded series of S-nitroso-N-acetyl-DL-penicillamine (Sigma, St Louis, Missouri, USA) concentrations, from 1×10^{-3} mol/L to 1×10^{-5} mol/

L. Acetylcholine (20 μg/kg BW; Daiichi Sankyo Co., Ltd, Japan) or saline was intravenously injected from femoral vein, and the change in potential was recorded.

The amount of acetylcholine-induced NO release in placebo group was expressed as 100 %; the NO release in other groups was expressed as percentage of that of control group.

2.8. Immunohistochemistry and histology

The heart was exposed, and a butterfly catheter was inserted into the left ventricle. The heart was flushed with 10 mmol/L PBS pH 7.4 for about 3 minutes, to remove all blood. Blood vessels then were fixed by perfusing 4 % paraformaldehyde in PBS (Wako Pure Chemical Industries Ltd., Osaka, Japan).

The following three area, aortic root, aorta, brachiocephalic artery were used in immunohistochemistry and histology. And data from brachiocephalic artery was shown. Blood vessels of interest were fixed in OCT compound (Tissue-Tek and SAKURA, Japan) and frozen by using a dry ice–acetone mixture, or liquid nitrogen. Frozen OCT-embedded tissue blocks were cut into 6-μm sections, which were placed on poly-L-lysine–coated microscope slides (Muto Pure Chemicals, Tokyo, Japan).

These slides were examined by using immunoperoxidase staining with anti-eNOS antibody (1:100, eNOS Rabbit PAb, Lab Vision Corporation, Fremont, California, USA) or an antibody to lectin-like oxidized low-density lipoprotein receptor 1 (1:50, LOX-1; anti-mouse LOX-1 monoclonal antibody, R&D Systems, Inc., Minneapolis, Minnesota, USA). Epitopes recognized by the primary antibody were visualized by labeling with streptavidin and biotinylated horseradish peroxidase (LSAB2 kit; DAKO, Kyoto, Japan) as described previously[15].

The slides were counterstained with Mayer's hematoxylin (LabVision, TA-125-MH). In histological investigation, cross-sections were obtained from at least three sections (front, middle, rear) of plaque. Vascular inflammation and calcification were confirmed by Miller's Elastica van Gieson (EVG) stain (Muto pure chemicals, Japan) and von Kossa stain (Modified Von Kossa, ScyTek, USA)[16], respectively.

2.9. Assay of total Cholesterol, prothrombin time, plasma nitrate and nitrite, and urinary 8-OHdG

Determinations were performed 12 weeks after the endpoint of experimental diet. Anticoagulated whole blood was collected from the abdominal aorta of mice under pentobarbital anesthesia by using Microtainer tubes (3.14 % sodium citrate; Becton Dickinson, Franklin Lakes, New Jersey, USA). Plasma was obtained after centrifugation at 1500 G for 15 minutes at 4 °C) and stored at –80 °C until analysis

Plasma total cholesterol level was determined enzymatically by using Wako reagents (Wako Pure Chemical Co) and Biomek 1000 (Beckman Coulter Inc, USA).

Prothrombin time was measured by using human thromboplastin (Thromborel S; Sysmex Corp., Kobe, Japan) in an automatic analyzer (Destiny Plus; Trinity Biotech, Wicklow, Ireland).

The levels of plasma nitrate or total nitrate/nitrite are measures of endogenous NO production. Plasma was prepared for NOx quantification by deproteinization by membrane ultrafiltration (Nano CEP Microsep Centrifugal Devices, Japan). To determine the nitrate/nitrite (NOx) levels, the plasma was incubated with reduced nicotinamide adenine dinucleotide phosphate (NADPH) and NADPH-dependent nitrate reductase to convert the nitrates present to nitrites. The nitrite level was then determined by using the Griess method and a commercially available analysis kit (NO_2 / NO_3 Assay Kit-C II, Dojindo, Japan). Concentrations were expressed in μmol/mL

Urine was collected without preservative in plastic containers, and stored at –80 °C until analysis of 8-OHdG. Levels of 8-OHdG in the samples were determined using an enzyme-linked immunosorbent assay (ELISA) (8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan). The assay was determined automatically (Biomek 1000, Beckman Coulter Inc., Brea, California, USA)

according to the manufacturer's instructions.

2.10. Statistical analysis

Results were expressed as mean ± SEM. Intergroup comparisons were made by using 1-way factorial ANOVA, followed by the Fisher PLSD test (Statview USA). Differences between means were considered significant at a *P* value less than 0.05.

3. Results

3.1. Body weight and food intake

Body weights did not differ between any of the groups of mice either before or after feeding of the high-fat experimental diets. The body weight after 18 weeks was 43.8 ± 1.6g in placebo group, 39.8 ± 1.8 g in warfarin group , 32.4 ± 0.54 g in control group. A significant difference was not admitted in each group.

The intake of food was 5.28 ± 0.14 g/day in placebo group, 5.45 ± 0.14 g/day in warfarin group, 5.22 ± 0.14 g in control group. Food intake did not differ between the placebo and control groups and between the warfarin and control groups.

3.2. Total Cholesterol

The cholesterol levels after 12 weeks feeding was 18.9 ± 1.24 mmol/L in placebo group, 20.0 ± 1.46 mmol/L in warfarin group. The total cholesterol levels in each group rose progressively over time to exceed 18mmol/L, compared to a mean value of 3.26 ± 1.58 mmol/L in control group.

Excepting control group, there was no significant difference statistically in between placebo and warfarin group.

3.3. Prothrombin time

Prothrombin time (PT, mean ± SEM) was measured after 12 weeks from the beginning of experimental diet.

PT values were 9.7 ± 0.1 seconds in placebo group, 14. 7 ± 1.1 seconds in warfarin group. INR values were 1. 98 in warfarin group.

3.4. Development of atherosclerosis

To assess the effect of warfarin on the development of atherosclerosis in mice fed a high-fat diet, we stained the aorta and major blood vessels with oil red O (which stains cholesterol) to calculate the lesional area as a percentage of total luminal area.

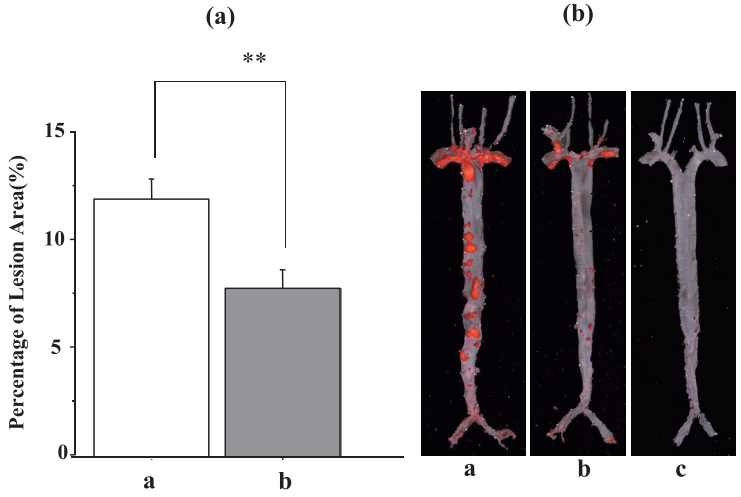

Fig.1 The effect of warfarin on the progression of atherosclerosis
(a): The comparison between placebo group and warfarin group in the extent of atherosclerosis after 12 weeks. a: Placebo group; b: Warfarin group; $n = 8$ in each group. **: $P = 0.008$
(b): Representative images of atherosclerotic regions identified by oil red O staining a: placebo group; b: warfarin group; c: control group (C57BL/6 mice); $n = 8$ in each group.

The extent of atherosclerosis (measured as a percentage of the total luminal area) after 18 weeks of feeding the various experimental diets was 12.0 % ± 1.0 % in the placebo group; 7.8 % ± 0.9 % in the mice that received warfarin (Figure 1a). The extent of atherosclerosis differed significantly between the placebo and warfarin groups (P = 0.008). Representative images of oil red O staining for atherosclerotic plaques are shown in Figure 1b.

3.5. Acetylcholine-induced NO release

The acetylcholine (20 μg IV/kg body weight)-induced NO release was 148.4 % ± 7.0 % (relative to that for the placebo group) in warfarin group. Compared with that of placebo group, NO release was significantly increased in warfarin group (P < 0.001) (Table 1).

3.6. Nitrate and nitrite (NOx) concentrations in plasma

Plasma NOx concentrations were 11.4 ± 0.9 μmol/L for placebo group, 19.7 ± 3.2 μmol/L for warfarin group. Compared with that in placebo group, the plasma concentration of NOx was increased in warfarin group (P<0.001) (Table 1).

3.7. eNOS expression

The expression of eNOS in endothelial cells was identified by immunohistochemistry. In cross-sections of the left brachiocephalic trunk, eNOS was specifically expressed in the endothelial cells of mice in the C57BL6 and warfarin groups but not in placebo group.

Table 1 Comparison among released NO, plasma nitrogen oxides, and urinary 8- OHdG after feeding apoE–/– LDLR–/– double-knockout mice a high-fat diet for 12 weeks.

	released NO [1)]	Plasma NOx	urinary 8-OHdG[2)]
Placebo group	100 %	11.4 ± 0.9 μmol/L	5.0 ± 1.3 ng/mg crea
Warfarin group	148.4 % ± 7.0 %**	19.7 ± 3.2 μmol/L**	2.7 ± 1.4 ng/mg crea**

Warfarin group:INR:1.98), n = 8 in each group. **,: P < 0.001
1):NO release was induced by treatment with acetylcholine at 20 μg/kg body weight; Each value:relative value for the placebo group
2):Each value was adjusted for the creatinine (Crea) concentration.

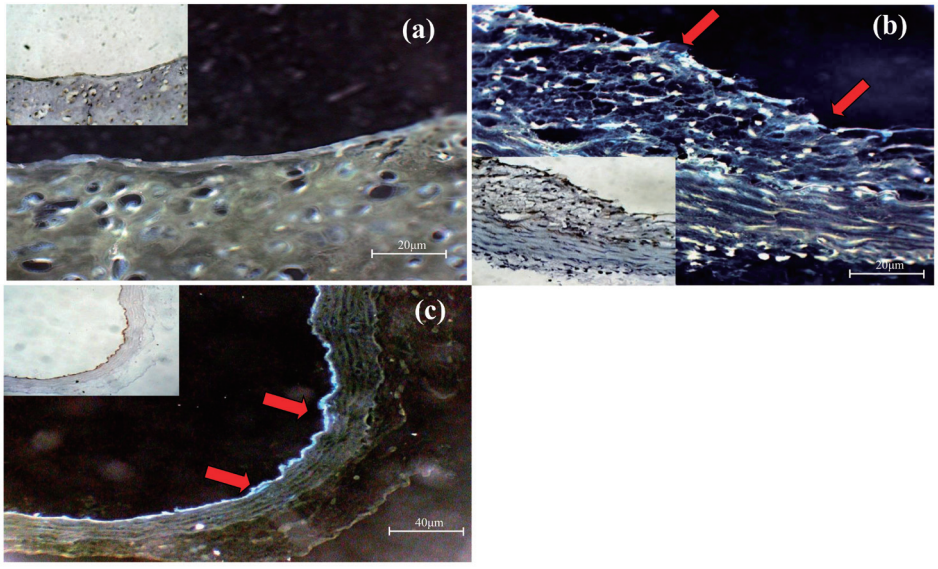

Fig. 2 eNOS expression determined by immunohistochemistry after 12 weeks
Data are shown as pseudocolored images of the original data (inset). a: placebo group; b: warfarin group; c: control group (C57BL6 mice); The brachiocephalic artery; Magnification: 200× (a, b), 100× (c); The arrow shows the positivity area.

3.8. Markers of oxidative stress

The concentration of urinary 8-OHdG was 5.0 ± 1.3 ng/mg creatinine in placebo group, 2.7 ± 1.4 ng/mg creatinine in warfarin group. The urinary 8-OHdG concentration was lower in warfarin group than in placebo group (Table 1).

3.9. LOX-1 expression

The expression of LOX-1 in endothelial cells was detected by immunohistochemistry. In cross-sections of the left brachiocephalic trunk, LOX-1 was specifically expressed in the endothelial cells of mice in placebo group. And was slightly expressed in warfarin group but not in control group (Fig.3).

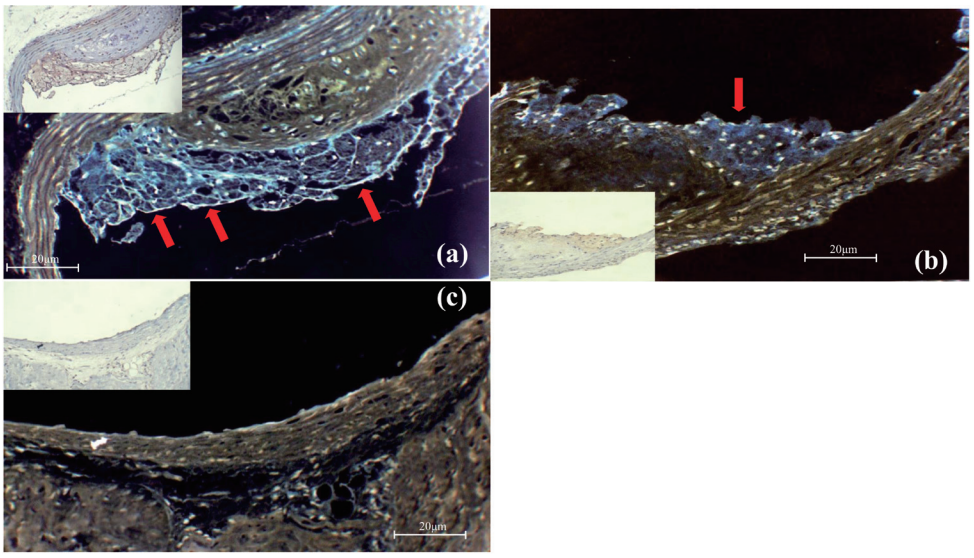

Fig. 3 LOX-1 expression determined by immunohistochemistry after 12 weeks
Data are shown as pseudocolored images of the original data (inset). a: placebo group; b: warfarin group; c: control group (C57BL6 mice); The brachiocephalic artery; Magnification: 200×, The arrow shows the positivity area.

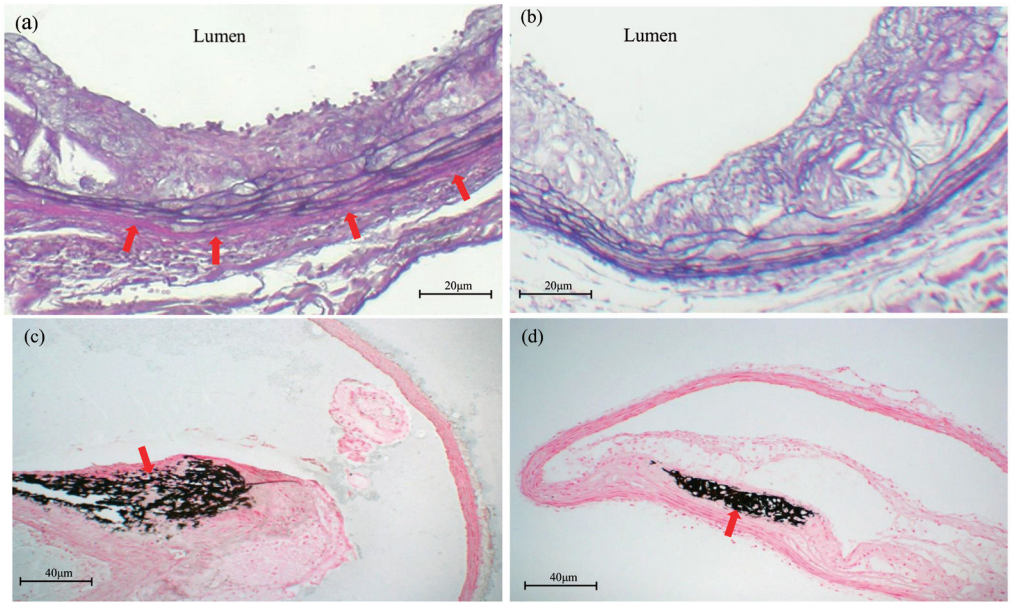

Fig. 4 Histology of plaque sections derived from both groups after 12 weeks
a,b: Elastica Van Gieson (EVG) stains; c, d: von Kossa stains, a, c:placebo group; b, d: warfarin group, a:The arrow shows collagen fiber layer, c, d: The arrow shows the positivity area. The brachiocephalic artery; Magnification: 200 × (a, b), 100 × (c, d)

3.10. Histology of plaque regio

Vascular inflammation and calcification were confirmed by Elastica Van Gieson (EVG) stains and von Kossa stain, respectively. The collagen fiber layer associated with the inflammation was observed at the lower layer of elastic lamina adjacently in the plaque derived from placebo group, but not in warfarin group (Fig 4, a, b).

The regions of loose/coiled elastin fibers and fragmented elastin fibers was confirmed in placebo group compared with warfarin group. Plaques formed in the same vascular area, were examined for vascular calcification in both groups. A significant difference in Ca-stain images was not admitted in both groups (Fig.4 c, d). A similar observation in vascular calcification was confirmed at other plaque areas.

4. Discussion

Atherosclerosis ultimately develops due to the deposition of LDL-cholesterol in the arterial wall[17]. However, atherosclerosis reflects contributions from diverse other processes, including oxidative stress, inflammation, hypercoagulability, and functional disorders of vascular endothelial cells[18-20].

Therefore, we have to understand that the progression of atherosclerosis not only due to dyslipidemia but also various thrombogenic atherosclerotic promoting factors. DKO mice used in the present investigations characteristically display a marked lipid metabolic disorder, progress severe hyperlipemia and are prone to atherosclerosis in the relatively short term[21,22].

Long-lasting peroral administration of warfarin (INR: 1.98) reduced the progression of atherosclerosis in DKO mice. The total cholesterol level in each groups rose progressively over time to exceed 18 mmol/L after 12 weeks feeding. It is not generally noted that warfarin lowers the level of total cholesterol like a statin. In this study, it was not recognized difference in both groups. The lowering of cholesterol level is essential in the prevention of atherosclerotic progression. However the lowering of cholesterol level was not confirmed in warfarin groups. Further studies are necessary to investigate the relationship between the level of cholesterol and the progression of atherosclerosis.

Whereas oil red staining revealed plaque augmentation in DKO mice fed a high-fat diet, lipid deposition was markedly reduced in mice fed a similar diet but containing warfarin.

We therefore investigated the expression of LOX-1[23], which is important in the uptake of oxidized LDL[24].

LOX-1 is a scavenger receptor on endothelial cells and, unlike SR-A[25], preferentially recognizes. And its expression is induced by oxidized LDL rather than acetylated LDL[26,27]. Immunochemical staining revealed that LOX-1 was expressed slightly in the warfarin-treated mice. However its expression was lower compared with placebo group. These results obtained in DKO mice indicate that oxidized LDL uptake was upregulated through LOX-1 in placebo group compared with warfarin group. One possible explanation for the expression decrease in warfarin group, is that thrombin F-Xa increases the expression of LOX-1[28,29].

In a recent report on the several thrombin functions, thrombin increases the expression of LOX-1, and the effect is also found in activated F-X[28]. Therefore, it is suggested that the administration of vitamin K antagonist prevents the production of thrombin and activated factor-X, in association with atherosclerosis progression, and led to decrease of LOX-1 expression. In addition, oxidized LDL upregulates the expression of LOX-1[30]. Therefore, DKO mice have a potential for oxidization of LDL.

The production of active oxygen species (free radical) is expected to increase simultaneously, if the concentration of plasma oxidized LDL is high level. We therefore measured the levels of 8-OHdG in urine as an index of oxidative stress and found that 8-OHdG in the placebo group was increased significantly compared with that in warfarin group, reflecting decreased oxidative stress due to warfarin treatment.

These data support the LOX-1 results, and suggest that several oxygen species have a close relation to the progression of atherosclerosis in DKO

mice. Perhaps the luminal surface of endothelial cells is particularly sensitive to damage from excessive concentrations of free radicals because of its continuous exposure to the blood flow[31].

In this way, increased levels of oxidative stress increase the damage to endothelial cells and contribute to the progression of atherosclerosis. In contrast, treatment with warfarin apparently decreased oxidative stress. Whether the inhibition of vitamin K dependent coagulation factors directly decreases oxidative stress is unknown.

However, there is a possibility that the expression of iNOS induced by thrombin was prevented. Thrombin induces the expression of iNOS and increases the inflammation[32-34]. iNOS generates excess NO, and it changes into peroxy nitride (·ONOO2-), which is an active free radical. Peroxy nitride increases in vivo oxidative stress. It is necessary to investigate the influence on the iNOS expression by the warfarin administration. Endothelial cells play a central role in maintaining hemostasis in the circulation[35,36]. Because endothelial dysfunction decreases the amount of NO released from these cells[37], we assessed acetylcholine-induced NO release in vivo as a marker of endothelial cell function. The amount of NO released and blood levels of NOx were increased significantly in warfarin-treated group compared with placebo group. Therefore, the treatments with warfarin led to the improvement of endothelial function associated with the repression of atherosclerotic progression. The release of NO from endothelial cells requires the expression of eNOS at the cell surface[37]. Immunohistochemistry revealed the expression of eNOS in control and warfarin group. The expression in placebo group was found slightly compared with other groups. These data indicate that vitamin K dependent coagulation factors promoted the down-regulation of eNOS expression. According to a study using human umbilical vein endothelial cells, thrombin stimulation significantly down-regulated the eNOS protein level[38].

It seems that thrombin prevents endothelial-cell-dependent relaxation. Treatment with warfarin to inhibit vitamin K dependent coagulation factors in DKO mice decreased the down-regulation of eNOS expression.

These data suggest that vitamin K dependent coagulation factors related enzymes generating thrombin might also prevent the expression of eNOS. This might lead to promote the damage of circulation, and assist the progression of atherosclerosis.

Furthermore, a recent report on the use of vitamin K antagonist has indicated that vitamin K antagonist induces the development of vascular calcifications[39-41]. The anticoagulation treatment with vitamin K antagonist leads to systemic calcification at the same time, and there is a possibility to bring the result of conflicting with atherosclerotic progression.

Plaques formed in the same vascular area, were examined for vascular calcification in both groups. A significant difference in Ca-stain images was not admitted in both groups.

We considered the possibility of not inducing a significant calcification because the warfarin dosage is comparatively low (INR: 1.98). A further examination is necessary to clarify the relation between the vitamin K dependent factors and the progression of arteriosclerosis. Furthermore, EVG stains revealed some common characteristics of vessel tissue. The collagen fiber layer by inflammation was observed significantly in the vessel with plaque derived from placebo group. Severity of inflammation in the plaque proximal was not same at both groups. Vascular tissue in the plaque proximal derived from placebo group unlike warfarin group had large collagen fiber layer. A possible explanation is that warfarin prevented the production of thrombin, thereby suppressing production of inflammation substances derived from thrombin[42,43].

In this study, the progression of atherosclerosis, together with the improvement of endothelial function and the reduction of oxidant stress, was diminished by warfarin in DKO mice.

In conclusion, our results showed that the generation of thrombin was inhibited by administration of warfarin (INR: 1.98), and various bioavailability of thrombin was also inhibited, and it

led to the reduction of atherosclerotic progression. However, it notes that this report is a result in the limited condition and the investigation of other vitamin K dependent proteins to be influenced with vitamin K antagonist has not been performed.

Disclosure of Conflict of Interest

The authors have no conflicts of interest.

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