Antithrombotic Effect of Oral Administration of Mozuku (Cladosiphon okamuranus, Brown Seaweed) Extract in Rat

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Summary Dysfunction of vascular endothelial cells causes the risk of thrombosis. Aim of this study is to evaluate the antithrombotic effect of Okinawa mozuku (brown seaweed, Cladosiphon okamuranus) extract by using cultured vascular endothelial cells and rat carotid arterial thrombosis model induced by ferric chloride (FeCl₃). The cell line (TKM-33) established from human umbilical vein endothelial cells were cultured with or without Okinawa mozuku extract. After incubation for 24 h, the conditioned medium was collected to evaluate urokinase-type plasminogen activator (u-PA) activity. Next, rats were fed with water or water containing 5% of Okinawa mozuku extract for 8 wk. After 8 wk of treatments, the rats were provided for the carotid arterial thrombosis model, and fibrinolytic factor and coagulation factor in blood were measured. Okinawa mozuku extract significantly augmented u-PA activity in the conditioned medium. The decrease of carotid artery blood flow induced by 40% FeCl₃ injury in rats fed with Okinawa mozuku extract was less than that in control rats. Thus, oral administration of Okinawa mozuku extract prevented thrombus formation in this model. Oral administration of Okinawa mozuku extract significantly increased u-PA activity in euglobulin fraction, compared with control group. On the other hand, platelet aggregation activity, activated partial thromboplastin time, and active PAI-1 level in plasma exhibited no significant differences between control and Okinawa mozuku groups. These results indicate that oral administration of Okinawa mozuku enhances fibrinolytic activity in plasma and prevents thrombus formation which is induced by injury of vascular endothelial cells.

Key Words Okinawa mozuku, thrombosis, plasminogen activator, fibrinolytic activity, vascular endothelial cells

Vascular endothelial cells (VEC) play a major role in maintaining blood flow through their antithrombogenicity. Anticoagulation and fibrinolysis are involved in the antithrombogenicity of VEC. The anticoagulation characteristics of VEC depend on heparan sulfate proteoglycan (HSPG) and thrombomodulin (TM) existing on endothelial cell surface. HSPG binds with tissue factor pathway inhibitor or antithrombin III, and inhibits coagulation cascade (1, 2). While, after binding of TM with thrombin, this complex activates protein C and inhibits coagulation cascade, subsequently (1). Also, the antithrombotic characteristics of VEC depend on self-produced fibrinolytic factors, such as tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA) and inhibitor of plasminogen activator (PAl-1). Plasminogen activator activates plasminogen to plasmin that has degradation activity of fibrin, which is a main component of thrombus. Normally, fibrinolysis and coagulation have been balanced well. However, the dysfunctions of VEC due to oxidative stress, hyperlipidemia, hyperglycemia, hypertension or inflammation cause the risk of thrombosis (3–6). Thrombosis develops thrombotic diseases, such as myocardial infarction and cerebral infarction. In order to prevent or ameliorate thrombosis and its related diseases, it is considered that investigations of food or food ingredients for maintaining or activating VEC’s function such as fibrinolytic system are important and beneficial.

It was reported that the brown seaweeds had various nutraceutical and pharmacological functions including anti-oxidative, anti-inflammatory, antiangiogenic, anti-hyperlipidemic and anticoagulant activity (7–11). Fucoids, sulfated polysaccharides, are rich in brown seaweeds and are like heparin. Some in vitro studies showed that fucoids from brown seaweeds inhibited...
thrombotic activity (12) and prolonged activated partial thromboplastin time (APTT) (13). Furthermore, intravenous administration of sulfated galactofucan from brown seaweed showed the antithrombotic activity in the rat vena cava ligation thrombosis model (14). Thus, polysaccharides from brown seaweed might be useful for prevention of thrombosis via anticoagulation. However, the effect of polysaccharides from brown seaweeds on fibrinolysis is not well clarified.

Okinawa mozuku (brown seaweed, Cladosiphon oka-muranus) is very popular in Japanese cuisine. Okinawa mozuku abundantly contains fucoidan and it is reported that the fucoidan from Okinawa mozuku is absorbed through the small intestine in vitro and in vivo (15). However, the anticoagulant effects of Okinawa mozuku were not shown (12, 13). Moreover, the effects of oral administration of Okinawa mozuku on the fibrinolysis were not clear. In this study, we investigated the fibrinolytic effects of Okinawa mozuku extract by using cultured VEC and the antithrombotic effects of oral administration of it by using rat carotid arterial thrombosis model induced by ferric chloride (FeCl₃).

MATERIALS AND METHODS

Preparation of Okinawa mozuku extract. Okinawa mozuku (Cladosiphon oka-muranus) was purchased from commercially available products (Iki mozuku; Itosan Co. Ltd., Okinawa, Japan). Mozuku and distilled water of its commercially available products (Iki mozuku; Itosan Co. Ltd., Okinawa, Japan). Mozuku and distilled water of its equal weight were mixed and autoclaved for 15 min. After cooling at room temperature, it was centrifuged at 1,200 × g for 10 min and supernatant was collected as Okinawa mozuku extract and stored at −20°C until using experiments.

Cell culture. The cell line (TKM-33) that was established from human umbilical VEC (HUVEC) (16) were used. The cells were plated onto 24 well plate (3×10³ cells/well) and cultured in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA). After reaching subconfluence, the medium was changed to FBS-free RPMI-1640 with or without 1% of Okinawa mozuku extract. After incubation for 24 h, conditioned medium and cell lysate were collected for assay of u-PA activity.

Assay of u-PA activity with fibrin zymography. u-PA activity was measured by fibrin zymography as described elsewhere (17). Briefly, protein samples were separated by 10% polyacrylamide gel contained 0.55 mg/mL bovine plasminogen-rich fibrinogen (Organan Teknika, Dublin, Ireland) and 0.056 U/mL thrombin (Midori Juji, Osaka, Japan), subsequently soaked in 2.5% Triton X-100 solution for 60 min, and then incubated in reaction buffer (0.1 mol/L glycine-HCl, pH 8.4) at 37°C for 18 h. After that, the gel was stained with Coomassie Blue G-250 (FUJIIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 30 min and destained with multiple changes of destain solution (44% methanol, 11% acetic acid) until lysis bands appeared.

Animal experiments. All animal experimental procedures were approved by the Kindai University Experimental Animal Committee (approval number: KAAG-26-009) and followed by the animal experiment guideline of Kindai University. Male Sprague-Dawley rats of 5 wk of age were purchased from CLEA Japan, Inc. (Tokyo, Japan). The rats had been freely fed and given water while they were kept in an environment with a 12 h light/dark cycle at 22±2°C. After acclimatization, the rats were divided into two groups (Okinawa mozuku [5% of Okinawa mozuku extract in drinking water] and control [drinking water only]). After 8 wk of treatments, the rats were provided for the carotid arterial thrombosis model. The blood samples were collected for measurements of platelet aggregation activity, activated partial thromboplastin time (APTT), u-PA activity in euglobulin fraction, plasma active PAI-1 levels.

Preparation of the euglobulin fraction. Plasma (0.5 mL) was mixed with 9.5 mL of 0.016% acetic acid in test tube, and then it placed on ice for 20 min and centrifuged at 2,000 × g at 4°C for 15 min. The supernatant was discarded and the precipitated euglobulin fraction was resuspended in 0.4 mL of 309 mmol/L NaCl containing 5.3 mmol/L barbital buffer (pH 7.4). The euglobulin fraction was stored at −80°C until using for zymography.

The carotid arterial thrombosis model. Rats were anesthetized by sodium pentobarbital (50 mg/kg, i.p.) and the right carotid artery was exposed. The filter paper (10×5 mm) was placed under the right carotid artery. FLO-C1 (OMEGAWAVE, Inc., Tokyo, Japan) probe was attached to the carotid artery to monitor blood flow. Then, vascular injuries were made by applying 40% FeCl₃ on a filter paper. After FeCl₃ application, carotid artery blood flow (mL/min/100 g) was monitored continuously for 30 min.

Measurement of platelet aggregation. The blood sample was centrifuged for 10 min (300 × g at 4°C) and supernatant was collected as platelet rich plasma (PRP). After collecting PRP, the blood samples were subjected to further centrifugation for 15 min (1,200 × g at 4°C) and supernatant was collected as platelet poor plasma (PPP). Platelet aggregation in PRP was initiated by 1 mg/mL of collagen reagent Horm® (Moriya Sangyo K.K., Tokyo, Japan) and monitored using platelet aggregation analyzer, TPA-4C (Tokyo Photoelectric Co., Ltd., Tokyo, Japan).

Activated partial thromboplastin time (APTT), PAI-1. APTT was measured using commercial APTT kit (Thromborecheck APTT; Sysmex, Hyogo, Japan). APTT reagent (0.1 mL) and 0.1 mL of PPP were mixed in test tube and warmed at 37°C for 2 min. Then, pre-warmed (37°C) 0.1 mL of 0.02 mol/L CaCl₂ was added and coagulation time was measured. Active PAI-1 level was measured using commercial ELISA kit (INR, MI, USA).

Statistics. All statistical analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and the add-in software Statcel 3 (OMS Publishing Inc., Saitama, Japan). The data were shown as means±SD for each group. Statistical analysis was performed by Student’s t-test. All results were considered
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RESULTS

Effect of Okinawa mozuku extract on cultured vascular endothelial cells

To assess the effect of Okinawa mozuku extract on fibrinolytic activity of VECs in vitro, after incubation of VECs with or without Okinawa mozuku extract the u-PA activities in the conditioned medium and the cell lysate of them were measured by using fibrin zymography. Okinawa mozuku extract significantly increased u-PA activity in the conditioned medium of TKM-33 cells (Fig. 1A). In contrast, u-PA activity in the cell lysate was significantly lower in Okinawa mozuku extract, compared with control (Fig. 1B). These results suggested that Okinawa mozuku extract enhanced release of u-PA from inside of endothelial cells to outside.

Body weight, food intake, and water intake

Since Okinawa mozuku extract enhanced u-PA activity in vitro, we investigated the effect of oral administration of Okinawa mozuku extract on antithrombotic activity in rats. There were no significant differences in body weight, food intake and water intake between control and Okinawa mozuku groups (Table 1).

Effect of Okinawa mozuku extract on platelet aggregation activity and APTT

In order to assess the effect of Okinawa mozuku extract on blood coagulation system, platelet aggregation activity and APTT were measured. No statistically significant differences in platelet maximum aggregation rate, appearance time of maximum aggregation rate, and the area under the curve (AUC) for platelet aggregation were observed between control and Okinawa mozuku groups (Table 2). APTT measured in Okinawa mozuku group did not significantly differ from that in control group (Fig. 2).
Effect of Okinawa mozuku extract on FeCl₃-induced carotid artery thrombosis model

To evaluate the preventive effect of Okinawa mozuku on thrombosis, we examined the effect of oral administration of Okinawa mozuku extract in carotid artery thrombosis model induced by 40% FeCl₃ injury. Prior to generate thrombosis, rats were fed with or without Okinawa mozuku extract for 8 wk. Application of 40% FeCl₃ to carotid artery resulted in thrombosis and blood flow was gradually decreased. However, the decrease of blood flow in Okinawa mozuku group was less than that in control group (Fig. 3A). The area under the curve for blood flow in Okinawa mozuku extract group was significantly higher than that for control group. Control, n=4; Okinawa mozuku, n=5 were analyzed. Values are shown as mean±SD. *p<0.05, **p<0.01.

Discuss the results obtained from the Okinawa mozuku extract study.
or t-PA deficient mouse (20). Moreover, the recanalization rate of occluded artery in u-PA deficient mouse was lower than in WT mouse or t-PA deficient mouse (20). Therefore, it is thought that u-PA is more important for early blood clot resolution and vascular recanalization than t-PA. The augmented release of u-PA from endothelial cell by Okinawa mozuku may contribute to prevent thrombus formation and promote clot lysis.

In order to confirm the effect of oral administration of Okinawa mozuku on thrombus formation, FeCl3-induced arterial thrombosis model was used. FeCl3-induced thrombosis model has been well used as the experimental arterial thrombosis model (21–23). In this model FeCl3, an oxidizing agent, induces denudation of endothelial cells and exposes the subendothelium such as collagen. Then, coagulation cascade proceeds and thrombus is formed. The FLO-C1 has been used to evaluate thrombosed vessel blood flow (24, 25). It was demonstrated that Okinawa mozuku prevented the thrombotic occlusion due to FeCl3-induced carotid artery injury (Fig. 3A and B). Furthermore, the oral administration of Okinawa mozuku extract increased u-PA activity in plasma euglobulin fraction (Fig. 4A). This phenomenon was supported by the in vitro study (Fig. 1A). Therefore, administration of Okinawa mozuku extract may promote release of u-PA from endothelial cells. These findings confirmed that arterial thrombotic occlusion was prevented by Okinawa mozuku which enhanced u-PA activity in plasma.

No changes were observed in platelet aggregation and APTT between control group and Okinawa mozuku group (Table 2 and Fig. 2). These results suggest that Okinawa mozuku has neither the antiplatelet nor the anticoagulant property. Moreover, these results are consistent with the previous in vitro study (12).

PAI-1 is a main inhibitor of u-PA and regulates fibrinolytic system. PAI-1 blocks the conversion of plasminogen to plasmin via inhibition of u-PA activity. PAI-1 influences thrombus formation and degradation, and is a risk factor for arterial thrombosis (26). In the present study, oral administration of Okinawa mozuku extract did not affect plasma PAI-1 level (Fig. 4B). Therefore, it is considered that PAI-1 is not involved in the antithrombotic effect of Okinawa mozuku extract.

For thrombotic diseases, many attempts have been made to develop chemical compounds which would enhance t-PA release from endothelial cells (27, 28). However, these compounds have undesirable side effect. Therefore, Okinawa mozuku has attracted attention because of their safety. Furthermore, because of the previous study in which the time to occlusion after FeCl3-induced carotid artery injury in u-PA deficient mouse was shorter than t-PA deficient mouse as described above (20), it is speculated that u-PA releaser may be more efficient to prevent thrombus formation or promote clot lysis than t-PA releaser.

Since fucoidan is water-soluble, it is thought that Okinawa mozuku extract contains fucoidan. The absorption of fucoidan, which is viscos polysaccharide in blown seaweed, is reported (15, 29). Oral administration of fucoidan from Laminaria japonica prolonged the time to occlusion of blood vessel in electrical induced arterial thrombosis model. This phenomenon was accompanied with anticoagulation activity, downregulation of thromboxane B2, upregulation of 6-keto prostaglandin F1α, antiplatelet activity and effective fibrinolysis (29). The mechanism of the antithrombotic activity of fucoidan depends on its molecular weight (29). Although the main components of fucoidan are fucose and sulfate, fucoidan contains uronic acids and monosaccharides such as mannose, galactose, glucose and xylose (30). The chemical property of fucoidan and bioactivity of fucoidan differ depending on the origin of seaweed species, and have some relation with sulfate content and position, molecular weight and sugar composition (12, 30). In this study, Okinawa mozuku extract did not possess anticoagulation activity but enhanced fibrinolytic activity. These findings suggest that molecular weight and/or chemical property of fucoidan from Cladosiphon okamuranus are not similar to those from Laminaria japonica.

The limitation of this study is that components of Okinawa mozuku extract are unclear and effective ingredients are unknown. Because the Okinawa mozuku extract has high viscosity, it is considered that it is rich in fucoidan and alginic acid, which are the main viscous polysaccharides of Okinawa mozuku. Although it is reported that alginic acid prevents thrombin generation (31), the involvement of alginic acid in fibrinolysis is unclear. In order to clarify this involvement, further study will be need.

In conclusion, it was demonstrated that Okinawa mozuku extract possessed the ability to enhance u-PA activity through an increase in u-PA release from endothelial cells both in vitro and in vivo. Furthermore, we also showed the antithrombotic effect of oral administration of Okinawa mozuku extract on rat carotid arterial FeCl3-induced thrombosis model. It may be a beneficial food or supplement for the prevention of thrombosis and its related diseases.

REFERENCES


