Online publication ahead of print, AJMHS Vol 54, 2020

Mozuku (Cladosiphon okamuranus) a Brown Seaweed, Inhibits the Migration of Vascular Smooth Muscle Cells Co-cultured with Adipocytes

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Abstract

Background: The mortality rate of patients with arteriosclerosis-related vascular disease is extremely high. The central phenomenon that leads to the intimal thickening of arteries is the migration of vascular smooth muscle cells (SMCs) from the media to the intima and the subsequent proliferation of SMCs in the intima.

Aims: The aim of this study to establish the effect of Okinawa mozuku in preventing and ameliorating arteriosclerosis, it is necessary to first elucidate the effects of Okinawa mozuku on the transformation, proliferation, and migration of SMCs.

Study Design: This study was an in vitro experimentation, to evaluate the effect of the Okinawa mozuku extract on the migratory ability of SMCs in the presence of adipocytes.

Methods: This study examined the effect of the Okinawa mozuku extract on the migratory ability of vascular SMCs in the presence of adipocytes. For this purpose, we first investigated the effect of Okinawa mozuku extract on the lipid droplet content in adipocytes. The accumulation of lipids in the adipocytes was evaluated with Oil Red O staining. The Boyden chamber method was used to evaluate the effect of adipocytes on migratory ability of the SMCs. Briefly, the upper chamber in which SMCs were seeded was inserted to the lower chamber in which adipocytes were cultured. Therefore, we performed SMC/adipocyte co-culture (SACC) using the Boyden chamber. Migrated cells that passed

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through the membrane in the Boyden chamber were quantified on 0, 2, 4, 6, and 8 days of adipocyte differentiation. Moreover, in the adipocytokine assay, the effect of the Okinawa mozuku extract on the levels of active PAI-1 and the levels of adiponectin in the SACC medium were also measured.

Results: The results of this study demonstrated that the Okinawa mozuku extract suppressed the accumulation of lipid droplets in the mature adipocytes. Furthermore, the Okinawa mozuku extract suppressed the levels on active form of plasminogen activator inhibitor-1 (PAI-1), and increased the levels of the anti-inflammatory adipocytokine, adiponectin. Furthermore, using our original co-culture system (SACC), we investigated the effect of adipocyte on smooth muscle cell migration. The migratory ability of vascular SMCs in the presence of adipocytes increased as the adipocytes increasingly differentiated. The Okinawa mozuku extract decreased the levels on active form of PAI-1 in SACC, suppressing the migration of vascular SMCs.

Conclusion: Our study revealed that the Okinawa mozuku extract is effective in suppressing the progression of arteriosclerosis.

Key Words: the Okinawa mozuku, migration, vascular smooth muscle cells, adipocytes

INTRODUCTION

Metabolic syndrome is characterized by changes in the forms and functions of vascular walls and inflammatory cells. These changes are induced by humoral factors secreted from various sources, including the adipose tissue. The prevalence of obesity, which is associated with metabolic syndrome, has increased in recent years, and it is currently estimated that approximately 1.9 billion people are obese worldwide (1, 2). In addition, the mortality rate of patients with arteriosclerosis-related cardiovascular diseases is extremely high. In fact, these diseases are the leading cause of death in developed countries, with 16.7 million deaths worldwide each year (3-5).

The walls of arteriosclerotic arteries are characterized by intimal thickening, remodeling, and hardening due to the loss of elasticity resulting from interactions between the endothelial cells, smooth muscle cells (SMCs), and inflammatory cells. This causes luminal narrowing and a decline in vascular function. The central phenomenon that leads to the intimal thickening of arteries is the migration of vascular SMCs from the media to the intima and the subsequent proliferation of SMCs in the intima (6).

Previous studies have reported that various types of cells secrete a variety of pro-migratory and growth factors that partake in this phenomenon in a sequential manner (7, 8). The factors that induce inflammation in the walls of the arteries during arteriosclerosis include direct physicochemical stress on the artery walls, metabolic stress, and the effects of various humoral factors. Of these, the involvement of adipocytokines, produced by adipocytes, has received attention in recent years. Hypertrophic adipocytes secrete large quantities of harmful cytokines, including the primary inhibitor of plasminogen activator (PA), PA inhibitor-1 (PAI-1), and tumor necrosis factor-α (9-11). Adipocytes also secrete the protective adipocytokine adiponectin, which is an anti-inflammatory cytokine. Secretion of adiponectin decreases with an increase in the quantity of accumulated lipid droplets in adipocytes (12).

Recent studies have demonstrated that the adipocytokines released from adipocytes have significant effects in maintaining homeostasis in the living body. Numerous studies, including our previous report, have focused on identifying various ingredients in the diet that regulate the production of adipocytokines (13). Of these, brown seaweeds, including mozuku, exhibit nutritional and pharmacological properties, including antioxidative, anti-inflammatory, antiangiogenic, antihyperlipidemic, and anticoagulant activities. These actions are thought to be mediated by fucoidan and fucose, which are the main components of mozuku. Fucoidan is a sulfated polysaccharide with heparin-like functions found in brown algae, and occurs abundantly in brown seaweed. Some *in vitro* studies have demonstrated that fucoidan isolated from brown algae suppresses thrombotic activity (14) and prolongs the activated partial thromboplastin time (15). Furthermore, intravenous administration of sulfated

galactofucan isolated from brown algae has been shown to have antithrombotic activity in a rat model of thrombosis, induced by ligation of the inferior vena cava (16). We previously demonstrated that administration of Okinawa mozuku extract suppresses thrombus formation in rats (17). Our results indicated that the anticoagulant effects of polysaccharides isolated from brown algae may help in preventing thrombosis.

Conversely, it has been reported that the fibrinolytic tissue-type PA (t-PA) and urokinase-type PA (u-PA), both of which are target enzymes of PAI-1 in vascular SMCs, are expressed in atherosclerotic lesions in human subjects (18-20). PAs are serine proteases that convert the inactive proenzyme plasminogen to its active form, plasmin. Since PAs is expressed in the lesions of atherosclerosis, it is inferred that it plays an important role in the progression of arteriosclerosis.

We hypothesized that Okinawa mozuku extract affects adipocytes, regulates adipocyte-derived PAI-1, adiponectin, and PAs, and suppresses the progression of arteriosclerosis. To establish the effect of Okinawa mozuku in preventing and ameliorating arteriosclerosis, it is necessary to first elucidate the effects of Okinawa mozuku on the transformation, proliferation, and migration of SMCs. In this study, we elucidated the effect of the Okinawa mozuku extract on the migratory ability of vascular SMCs in the presence of adipocytes. We also investigated the benefits of treatment with the Okinawa mozuku extract in suppressing the progression of arteriosclerosis, with the aim of elucidating its therapeutic potential in preventing thrombosis and related diseases.

MATERIALS AND METHODS

Preparation of Okinawa mozuku extract

Commercially available mozuku (*Cladosiphon okamuranus*) was purchased for this study (Iki mozuku; Itosan Co. Ltd., Okinawa, Japan). Mozuku and an equal weight of distilled water were mixed. The mixture was then centrifuged at 1,200 × *g* for 10 min, and the supernatant containing the Okinawa mozuku extract was collected. The supernatant was sterilized with a 0.2 μm pore filter (Millipore, MA, USA), and stored at -20°C until further experimentation.

Reagents

Oil Red O was purchased from Wako Pure Chemical Industries (Osaka, Japan). Antibodies against extracellular signal-regulated kinase (ERK)1/2 (K-23), phosphorylated (p-)ERK 1/2 (sc-16982), and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase conjugated anti-mouse IgG antibody was purchased from Southern Biotech (Birmingham, AL, USA). Bovine fibrinogen, containing trace amount of plasminogen, was purchased from Organon (Organon Teknika, Dublin, Ireland), and bovine thrombin was purchased from Mochida (Tokyo, Japan). A murine PAI-1 activity assay kit was purchased from Molecular Innovation (Novi, MI, USA; lot no. MPAIKT-910) and was used

according to the manufacturer's instructions. A mouse adiponectin/Acrp30 assay kit was purchased from R&D Systems (Minneapolis, MN, USA; lot no. 278969) and was used according to the manufacturer's instructions. All other reagents and chemicals were of the highest commercial grade.

Cell culture

Fibroblasts (3T3-L1 cells) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA; lot no. ATA31357), 200 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 100 mM pyruvic acid, at 37°C in a humidified atmosphere of 5% CO_2. Two days after reaching confluence, the cells were induced to differentiate into adipocytes by culturing in high-glucose DMEM supplemented with 10% FBS, 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine, and 2.5 μM dexamethasone for 2 days. After the stimulation, the 3T3-L1 cells were further cultured in high-glucose DMEM supplemented with 10% FBS and 10 μg/mL insulin for 8 days to induce the differentiation of the fibroblasts completely into adipocytes; during this period the culture medium was replaced every 48 h with fresh high-glucose DMEM supplemented with 10% FBS and 10 μg/mL insulin, with or without 1% Okinawa mozuku extract, 1% fucoidan (10 mg/mL, F5631, Sigma-Aldrich, St. Louis, MO, USA). Distilled water was used as the vehicle, which was added to the control culture. The 3T3-L1 adipocytes were subjected to further experimentation 2–8 days after the induction of differentiation. Briefly, 3T3-L1 adipocytes were fixed with lipid fixative solution, stained with Oil Red O, and washed with distilled water. The intracellular lipids were extracted with isopropanol. The extracted lipids were quantified at 492 nm on a microplate reader (357-00045T, Thermo Fisher Scientific, Waltham, MA). SMCs obtained from the thoracic aorta of the mice were prepared as previously described (21, 22), and cultured at 37°C in a humidified atmosphere of 5% CO_2.

Adipocytokine assay

To examine the effect of the Okinawa mozuku extract on the production of PAI-1 and adiponectin in adipocytes, we employed 3T3-L1 mature adipocytes cultured for 8 days after the induction of the differentiation of preadipocytes, as described above. The effect of the Okinawa mozuku extract on the levels of active PAI-1 and the levels of adiponectin in the adipocyte monocultures medium were investigated using PAI-1 and adiponectin assay kits, respectively.

PA assay

The activity of PA in the culture medium was measured by electrophoretic zymography as previously described (23). Briefly, protein samples were separated by 10% polyacrylamide gel contained 1.5 mg/mL plasminogen-supplemented fibrinogen and 10 NIH U/mL thrombin, after electrophoresis, 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 0.1% Coomassie Brilliant Blue G-250 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 30min, and destained with multiple changes of destain solution (44% methanol, 11% acetic acid) until lysis bands appeared. The activity of PA was determined by measuring the lytic area on the gel with a densitometer. The intensity of the band was quantified using Image Gauge Version 3.2 software.

Boyden chamber assay for assessing the migratory ability of SMCs

The Boyden chamber method was used to evaluate the effect of adipocytes on migratory ability of the SMCs. Briefly, the upper chamber in which SMCs were seeded was inserted to the lower chamber in which adipocytes were cultured. Therefore, we performed SMC/adipocyte co-culture (SACC) using the Boyden chamber. The membrane of the insert well had a pore size of 8 μm. The preadipocytes were seeded at a density of 4.5×10^3 cells/well in the lower chamber. The medium was changed every 2 days to a medium containing 1% Okinawa mozuku extract or vehicle (**Fig. 1A**).

The preadipocytes were induced to differentiate for 0, 2, 4, 6, and 8 days. The SMCs were seeded in the upper chamber at a density of 3×10^4

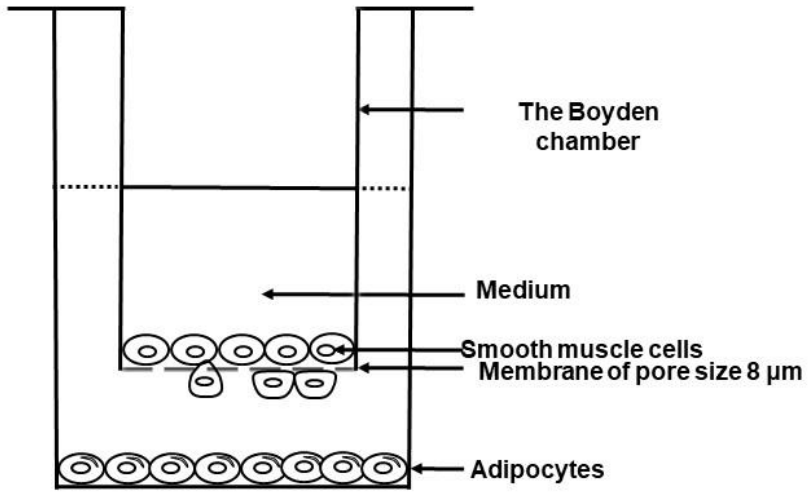

Fig. 1A Schematic illustration of the SACC assay using a Boyden chamber for evaluating the migratory capacity of SMCs

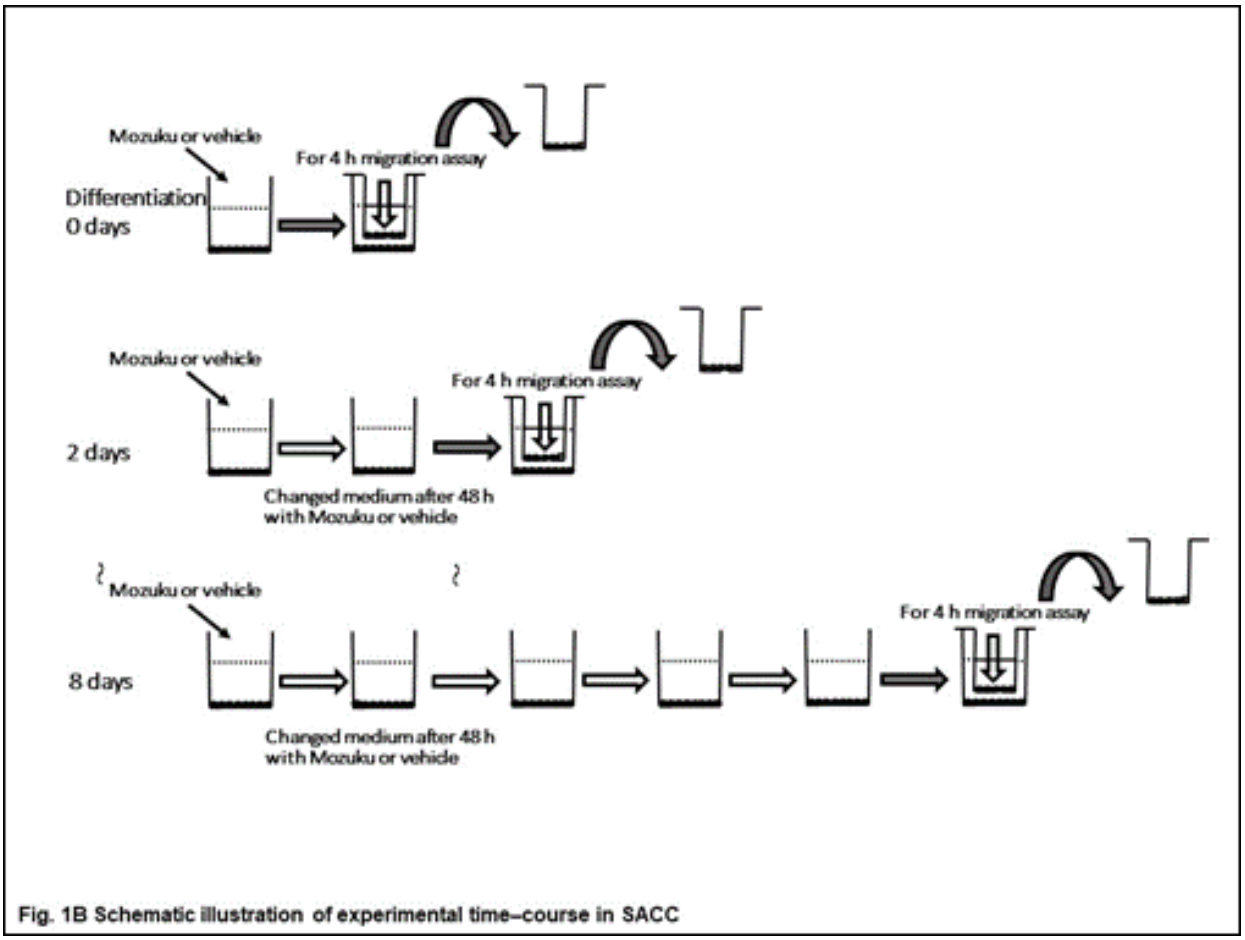

Figure 1. (A) Schematic illustration of the SACC assay using a Boyden chamber for evaluating the migratory capacity of SMCs. Pre-adipocytes were seeded at 4.5 × 103 cells per well in the lower chamber and induced to differentiate for 0, 2, 4, 6, and 8 days. SMCs were seeded in the upper chamber at 3.0 ×104 cells per well. Cells that migrated through the membrane were quantified. (B) Schematic illustration of the experimental time–course in the SACC. The migratory ability of SMCs in the SACC was evaluated after co-cultivation for 4 h by inserting the upper chamber into adipocytes with or without the Okinawa mozuku extract; medium was exchanged every 2 days. The migrating cell numbers of the adipocytes were measured for each specified differentiation day (0, 2, 4, 6, and 8 days) after 4 h co-cultivation.

cells/well. The migratory ability of the SMCs in SACC was evaluated after 4 h of co-cultivation by inserting the upper chamber into the wells. On the indicated days, the non-migrated SMCs were wiped with cotton to remove the SMCs in the non-migrating side of the upper chamber, subsequently the migrated SMCs were fixed and stained in the migrating side of the upper chamber using the Diff Quick Kit 2 (Sysmex, Kobe, Japan). The average number of migrating SMCs along the migrating side of the surface was counted from five randomly selected high-power fields using a 100× objective under a light microscope in three independent experiments (**Fig. 1B**). As shown in the adipocytokine assay, the effect of the Okinawa mozuku extract on the levels of active PAI-1 and the levels of adiponectin in the SACC medium were also

measured.

ERK signaling in SMCs

Two days after reaching confluence, the preadipocytes were induced to differentiate into adipocytes by culturing in high-glucose DMEM supplemented with 10% FBS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine, and 2.5 µM dexamethasone for 2 days. After the stimulation, the 3T3-L1 cells were further cultured in high-glucose DMEM supplemented with 10% FBS and 10 µg/mL insulin for 8 days to induce the differentiation of the fibroblasts completely into adipocytes; during this period the culture medium was replaced every 48 h with fresh high-glucose DMEM supplemented with 10% FBS and 10 µg/mL insulin, with or without 1% Okinawa mozuku extract.

Namely, culturing was performed every 2 days for 8 days in a differentiation-maintaining medium containing 1% mozuku extract.

After changing the medium in a differentiation maintenance medium containing 1% mozuku extract on the 8th day of differentiation, the cells were further cultured for 4 h. The culture medium and SMCs were seeded on the other plate. After the SMC was stimulated with the culture medium for 30 min, the medium was removed and the SMC was lysed in the cell lysis buffer.

The incubated SMCs were subsequently lysed for 20 min in 100 µL lysis buffer comprising 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM NaF, 0.1 mM Na_3VO_4, and 1 mM dithiothreitol. After incubating for 20 min, the cellular debris was removed by centrifugation at 14,000 × *g* for 10 min, and the supernatant was collected. The supernatant was used as a sample for analyzing phosphorylated ERK. The protein concentration was subsequently determined, and 20 µg of protein was mixed with an equal volume of 2× sample loading buffer and boiled for 5 min, following which the mixture was cooled on ice for 5 min. The total protein was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis and electro-transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in 1× Tris-buffered saline with Tween 20 (TBST), comprising 0.01 M Tris (pH 7.6), 0.1 M NaCl, and 0.1% Tween-20 for 1 h at 25°C with shaking, followed by incubation with anti-ERK and anti-p-ERK antibodies in TBST for 1 h. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 1 h. The immunoreactivity was visualized with an enhanced chemiluminescence system (Amersham Bioscience, Little Chalfont, UK). The density of the protein bands was quantified with Image Gauge Version 3.2 software (Fujifilm, Tokyo, Japan).

Statistical analyses

All experiments were performed in three independent replicates (n = number of independent experiments). The data are presented as the mean ± SEM and were analyzed by the Student's t-test, one-way analysis of variance,

and Dunnett's post-hoc test. P values < 0.05 were considered statistically significant.

RESULTS

The Okinawa mozuku extract and fucoidan inhibited the differentiation of adipocytes

In order to investigate the effects of the Okinawa mozuku extract on adipocyte differentiation, 3T3-L1 cells were induced to differentiate in the presence or absence of the extract for 8 days. The accumulation of lipids in the adipocytes was evaluated with Oil Red O staining. Treatment with the Okinawa mozuku extract and fucoidan significantly reduced the lipid droplet content in adipocytes that had been induced to differentiate for 8 days, compared to that of the untreated control cells. (**Fig. 2**).

The Okinawa mozuku extract suppressed the active PAI-1 and stimulated adiponectin secretion by adipocytes

We examined the levels of adipocytokines by the adipocytes in monoculture and found that the

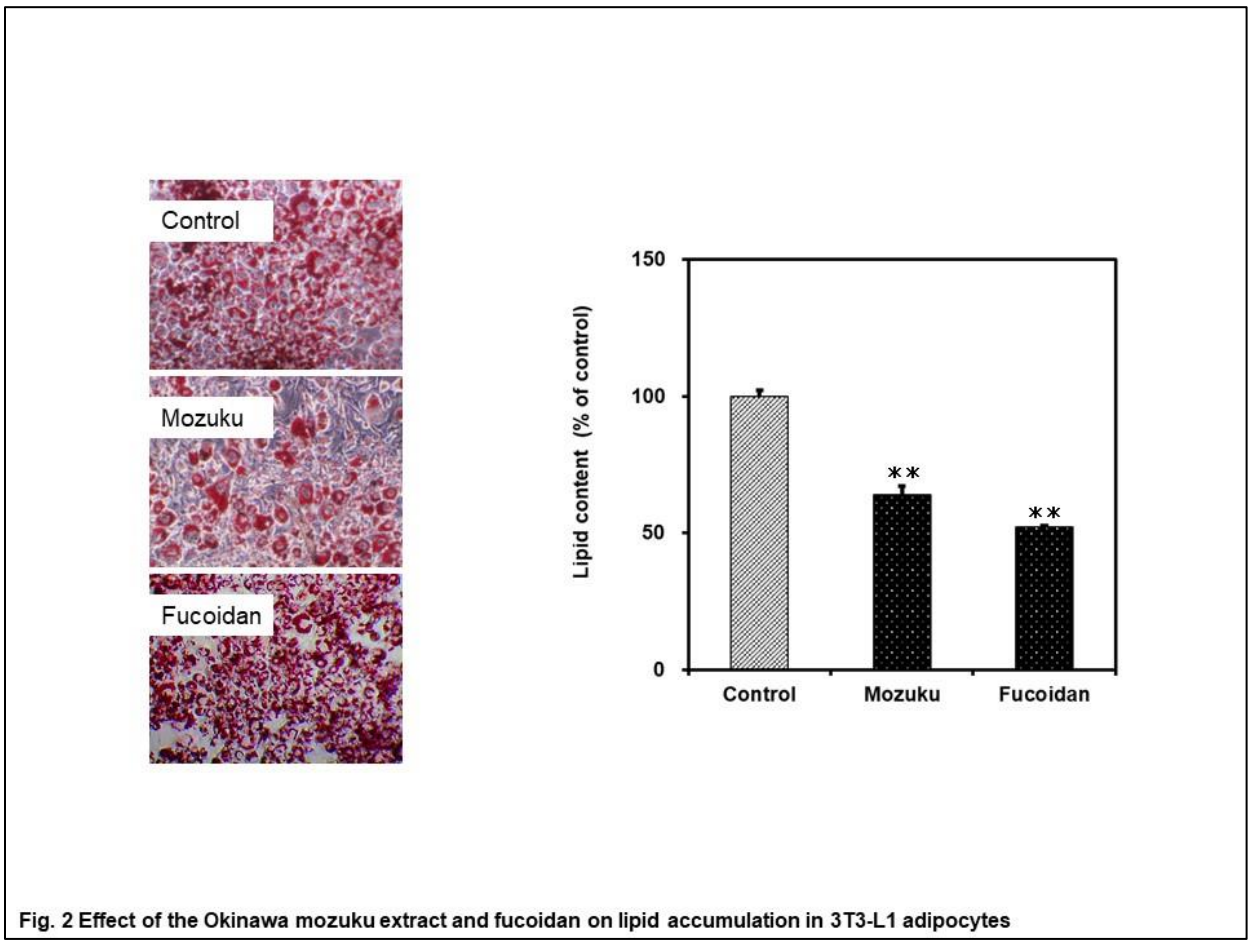

Fig. 2 Effect of the Okinawa mozuku extract and fucoidan on lipid accumulation in 3T3-L1 adipocytes

Figure 2. Effect of the Okinawa mozuku extract and fucoidan on lipid accumulation in 3T3-L1 adipocytes. Lipid content was assessed by Oil Red O staining, by measuring the absorbance of Oil Red O dissolved in isopropanol, at 492 nm. Cells were stained with Oil Red O at 8 days and examined microscopically (left, ×100). Differentiation was calculated as the percentage of cells with Oil Red O positivity relative to the control (right). Data are presented as the mean ± SEM of at least three independent experiments. ** P < 0.01 vs. 8 days of control.

Table 1. Active PAI-1 and adiponectin levels in adipocytes treated with the Okinawa mozuku extract after 8 days of adipocyte differentiation. Data are presented as the mean ± SEM of at least three independent experiments. # P < 0.05 vs. control.

Table 1 Active PAI-1 and adiponectin levels in adipocytes treated with the Okinawa mozuku extract after 8 days of adipocyte differentiation

	Active PAI-1 in culture medium of adipocyte (ng/ml)	Adiponectin in culture medium of adipocyte (ng/ml)
Control	6.66 ± 1.85	1.90 ± 0.48
Mozuku	1.76 ± 0.34#	4.19 ± 0.79#

Data are presented as the mean ± SEM of at least three independent experiments.
# P < 0.05 vs. control.

Okinawa mozuku extract significantly suppressed active PAI-1 and increased the levels of secreted adiponectin, compared to those in the untreated cells (**Table 1**).

Effect of the Okinawa mozuku extract on fibrinolytic factors

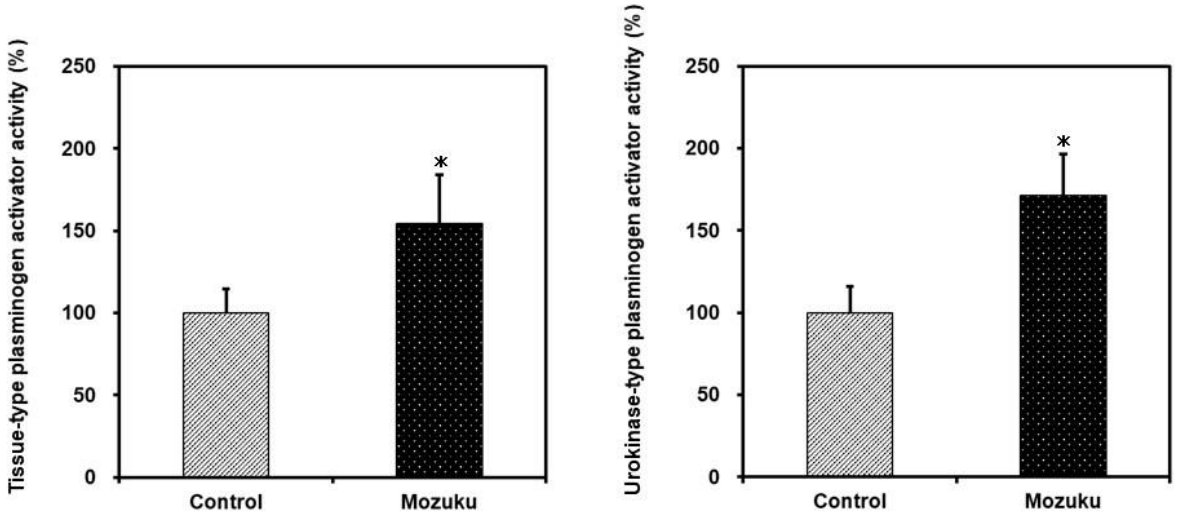

Figure 3. Effects of the Okinawa mozuku extract on tissue-type plasminogen activator and urokinase-type plasminogen activator in adipocytes after 8 days of adipocyte differentiation. The intensities of each lytic area were quantified as the percentage of the control mean in arbitrary units. Data are presented as the mean ± SEM of at least three independent experiments. * P < 0.05 vs. control.

The effect of the Okinawa mozuku extract on t-PA and u-PA activity in adipocytes was investigated after 8 days of differentiation. The activity of t-PA and u-PA secreted from the adipocytes was significantly higher following the addition of the Okinawa mozuku extract, compared to that from the control cells (**Fig. 3**).

The Okinawa mozuku extract inhibited migration of SMCs in the SACC

Analysis of the migratory ability of SMCs in the SACC revealed that the number of migrating SMCs that differentiated in the presence of adipocytes increased in a differentiation-dependent manner, with statistically significant differences observed at 4, 6, and 8 days (**Fig. 4**). Addition of the Okinawa mozuku extract to the co-cultures inhibited migration of the SMCs in a concentration-dependent manner, with significant reduction after 2, 4, 6, and 8 days of differentiation (**Fig. 5**).

Effect of treatment with the Okinawa mozuku extract on active PAI-1 and adiponectin levels in the SACC

We further examined active PAI-1 and adiponectin levels in the SACC following treatment with Okinawa mozuku extract. Active PAI-1 levels had significantly decreased and adiponectin levels had increased in the SACC after 8 days of differentiation under treatment with Okinawa mozuku extract compared to the

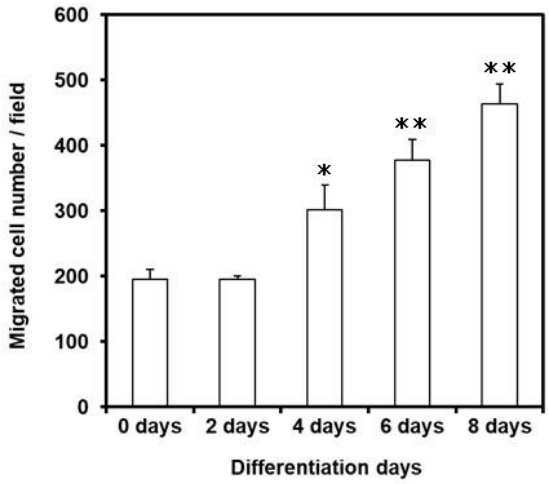

Figure 4. SMC migration induced by adipocyte differentiation. Migrated cells that passed through the membrane in the Boyden chamber were quantified on 0, 2, 4, 6, and 8 days of adipocyte differentiation. Data are presented as the mean ± SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. day 0 of differentiation.

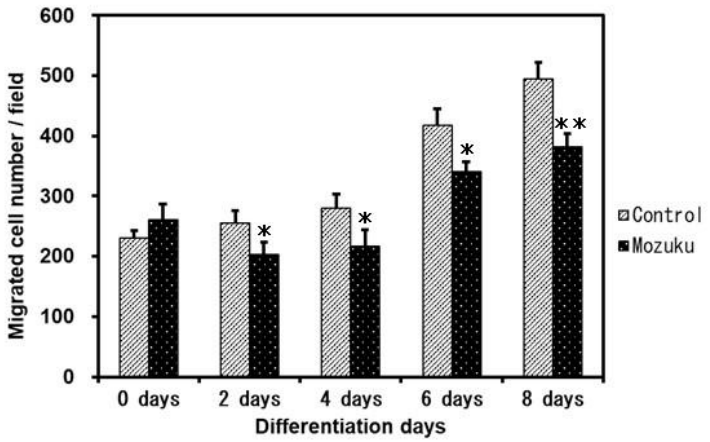

Figure 5. SMC migration in the presence of adipocytes stimulated with the Okinawa mozuku extract. Migrated cells that passed through the membrane in the Boyden chamber were quantified on 0, 2, 4, 6, and 8 days of adipocyte differentiation. Data are presented as the mean ± SEM of at least three independent experiments. * P < 0.05, ** P < 0.01 vs. control.

levels in the control medium (**Table 2**).

Table 2. Active PAI-1 and adiponectin levels in the SACC treated with the Okinawa mozuku extract after 8 days of adipocyte differentiation. Data are presented as the mean ± SEM of at least three independent experiments. # P < 0.05 vs. control.

	Active PAI-1 in culture medium of the SACC (ng/ml)	Adiponectin in culture medium of the SACC (ng/ml)
Control	1.31 ± 0.01	3.52 ± 0.42
Mozuku	1.27 ± 0.01#	3.89 ± 1.00

Data are presented as the mean ± SEM of at least three independent experiments.
# P < 0.05 vs. control.

Effect of the Okinawa mozuku extract on ERK signaling in SMCs

To elucidate the molecular basis underlying the inhibitory effect of the Okinawa mozuku extract on SMC migration, we further investigated the effect of the extract on ERK phosphorylation. For this, SMCs were stimulated for 4 h with the medium in which the adipocytes pretreated with

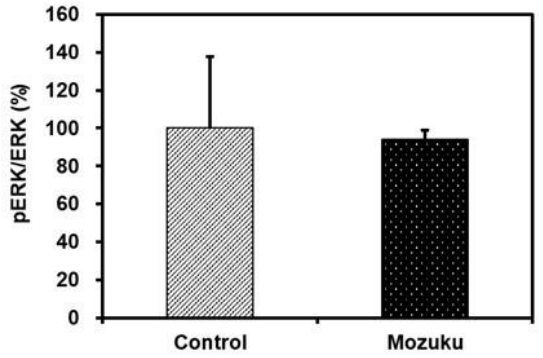

Figure 6. Effect of the Okinawa mozuku extract on ERK signaling in SMCs. SMCs were stimulated for 4 h with the medium in which the adipocytes pretreated with the Okinawa mozuku extract had been differentiating for 8 days. The expression of p-ERK in SMCs was evaluated by western blotting. The intensities of each bands were quantified as the percentage of the control mean in arbitrary units. Data are presented as the mean ± SEM of at least three independent experiments.

the Okinawa mozuku extract had been differentiating for 8 days. Results demonstrated that ERK phosphorylation in the SMCs was not affected by treatment with the Okinawa mozuku extract, compared to the control cells (**Fig. 6**).

DISCUSSION

In this study, we demonstrated that the Okinawa mozuku extract regulates the levels of adipocytokines from adipocytes and suppresses the migration of SMCs, which consequently suppresses arteriosclerosis at the initial stage of development.

The risk of developing arteriosclerosis and diabetes increases with the expansion of mature adipocytes. The results of this study demonstrated that the Okinawa mozuku extract suppressed the accumulation of lipid droplets in the mature adipocytes. Previous studies have demonstrated that oral administration of fucoidan, the main component of mozuku, caused weight loss in a rodent model of obesity (24, 25). It has been reported that these effects resulted from the characteristic effects of the Okinawa mozuku extract on the development and progression of obesity and diabetes. The present *in vitro* study on 3T3-L1 cells induced to differentiate into an adipocyte-like phenotype, suggests that the Okinawa mozuku extract may have delayed the process of preadipocyte differentiation into

enlarged, mature adipocytes.

Secretion of the adipocytokine PAI-1 increases with the enlargement of mature adipocytes, which accelerates lipid accumulation in these cells. It has been additionally reported that plasma levels of PAI-1 are high if insulin resistance is present along with obesity (26). A previous study demonstrated that *PAI-1* knockout mice suppressed weight gain (27). It has also been demonstrated that several PAI-1 inhibitors improved high fat diet-induced obesity in a murine pharmacological model (28, 29). The results of our study demonstrated that the Okinawa mozuku extract suppressed the active form of PAI-1, and increased the levels of the anti-inflammatory adipocytokine, adiponectin. This study demonstrated that the Okinawa mozuku extract reduced the accumulation of lipid droplets in the adipocytes and suppressed the active form of PAI-1, indicating that it suppresses adipogenesis.

There are two forms of the fibrinolytic factor PA, namely t-PA and u-PA. Both PA and PAI-1 are expressed in several organs in vivo. In particular, u-PA is recognized as a possible differentiation marker of 3T3-L1 cells and has been reported to play an important role in enhancing adipose tissue development during the induction of obesity [30]. Here, the Okinawa mozuku extract increased the activities of adipocyte-derived t-PA and u-PA. Although the significance of production of these fibrinolytic factors in adipose tissues remains to be determined, we hypothesize two reasons for this. First, the Okinawa mozuku extract could have decreased the levels of the active form of PAI-1 in adipocytes and prevented the binding of PA to PAI-1, resulting in enhanced activities of t-PA and u-PA. Second, the Okinawa mozuku extract separately played a role in decreasing the levels of the active form of PAI-1 and increasing the activities of t-PA and u-PA. However, the exact role of PA, plasminogen and plasmin in intimal thickening and atherosclerosis remain unknown. PA is involved in other physiological and pathological processes, as well as tumor cell invasion and metastasis [31-34]. In any case, the Okinawa mozuku extract increased PA activity and may have converted plasminogen to plasmin by increasing PA levels. Accordingly, this finding may also imply that the Okinawa mozuku extract dissolves fibrin, the main component of a thrombus, during thrombosis, which follows the progression of arteriosclerosis.

In this study, we examined the migratory ability of SMCs, which are components of early arteriosclerotic lesions. The migratory ability of SMCs is affected by various active substances secreted by the adipocytes. Using the SACC system, we investigated the effect of adipocytokines on the migratory ability of SMCs. Particularly, we exposed SMCs to various adipocyte-derived adipocytokines released at different stages of adipocyte differentiation. The migratory ability of vascular SMCs in the presence of adipocytes increased as the adipocytes increasingly differentiated. To our knowledge, this is the first demonstrated that migration of SMCs changes in the presence of

adipocytes. The results of our experiment revealed that changes in cellular chemotaxis depend on the differentiation of adipocytes and the migratory ability of SMCs, suggesting that regulation of adipocyte-derived adipocytokines is indispensable.

PAI-1 promotes or inhibits the migration of cells *in vitro* (35-46). However, the mechanisms underlying its activity remain to be elucidated (47). Our data suggest that PAI-1 causes cells to migrate; the anti-adhesive effect of PAI-1 is ascribed to its ability to compete for PA receptor and integrin binding to vitronectin. Moreover, the Okinawa mozuku extract decreased the active form of PAI-1 in SACC, suppressing the migration of vascular SMCs. Further investigations are required to confirm these findings.

Adiponectin suppresses insulin resistance, phosphorylation of intracellular ERK induced by insulin-like growth factor-1, and migration of SMCs (12, 48-52). However, in this study, we observed that the Okinawa mozuku extract had no effect on the levels of adiponectin in the SACC and did not affect ERK phosphorylation. However, in this study, we observed that the Okinawa mozuku extract had no effect ERK phosphorylation, despite the increased on the levels of adiponectin in the SACC.

Our results suggested that adiponectin may have a low contribution to the suppression of SMC migration by the Okinawa mozuku extract.

The limitations of this study are that the components of the Okinawa mozuku extract are not known and that the active components are yet to be identified. As the Okinawa mozuku extract is highly viscous, it is thought to be rich in fucoidan and alginic acid, which are the main viscous polysaccharides present in Okinawa mozuku extract. In this study, we observed that fucoidan, an adhesive sulfated polysaccharide covering the surface of seaweeds and abundantly found in brown algae, suppressed lipid accumulation in adipocytes. However, as fucoidan is a polymer, further examination is warranted, taking into consideration the rate of absorption of fucoidan into cells. It is also necessary to examine the effect of the extract on the migratory ability of the SMCs in the extracellular matrix, as well as the adhesion factors in the presence of adipocytes.

CONCLUSIONS

In conclusion, our study revealed that the secretion of adipocytokines alters the migratory ability of vascular SMCs. Furthermore, the Okinawa mozuku extract is effective in suppressing the progression of arteriosclerosis. The results of this study suggest that the Okinawa mozuku extract has beneficial effects in preventing thrombosis and related diseases.

Acknowledgements: None declared.

Conflict of Interest Disclosure: The authors declare that they have no conflict of interest.

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