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Effect of Chlorella Intake on the Development of Atherosclerosis and Spontaneous Thrombolytic Activity

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Abstract

Background and Purpose: Thrombotic disease is a leading cause of death in industrialized countries. The development of atherosclerosis is a major underlying pathogenesis. Atherosclerotic lesions are largely related to abnormalities in lipid metabolism, and improvement of dietary habits is of great significance. Chlorella is a unicellular organism belonging to the green algae family, and is consumed worldwide as a functional food for the purpose of health promotion due to its excellent nutritional balance including high quality protein. In this study, we investigated the effects of long-term consumption of Chlorella as a food on the development of atherosclerosis and its ability to dissolve thrombi caused by the disruption of the atherosclerotic layer as a functional study of Chlorella. **Methods:** ApoE$^{-/-}$ and Ldlr$^{-/-}$ double-knockout mice were fed a chlorella-supplemented experimental diet for 14 weeks. The Entire aorta method was used to measure atherosclerosis development, and the area of sclerotic vessels was evaluated as a percentage of the total area of vessels. In addition, mRNA levels of lipid metabolism-related proteins in the liver and blood vessels were analyzed, as well as blood lipoprotein analysis. Spontaneous thrombolytic activity was measured by measuring the change in volume over time of thrombus formed in microvessel running over the cremaster muscle of the mice using the He-Ne laser-induced thrombus model. **Results:** There was no significant difference between the two groups in atherosclerosis development compared to the placebo group. However, a significant decrease in SREBP-1 mRNA level and a significant increase in mRNA levels of LXR and CPY71a were observed in the chlorella group. Cholesterol and TG levels in each lipoprotein fraction did not differ between the two groups. On the other hand, thrombolysis *in vivo* was not significantly differ-

ent between the two groups in terms of thrombus volume at 60 minutes after thrombus formation. However, a trend toward decreased PAI-1 and TAFI mRNA expression levels was observed in the chlorella group. **Conclusion:** Intake of chlorella as a food suggested an effect on cholesterol catabolism, increased bile acid synthesis, improved lipid metabolism, and inhibited the development of atherosclerosis. Furthermore, it was suggested that chlorella may suppress the expression of fibrinolytic inhibitory factor and enhance thrombolytic activity.

Keywords

Chlorella, Atherosclerosis, Thrombolysis, SREBP-1, LXR, CYP71a, PAI-1, TAFI

1. Introduction

Atherosclerosis forms the underlying pathology of lifestyle-related diseases. It forms the basis of ischemic heart disease and cerebrovascular disease, and is one of the major causes of death in developed countries [1] [2]. The development and progression of atherosclerotic lesions are closely related to lifestyle habits, especially dietary habits. Therefore, improvement of daily dietary habits is of great significance. Many researchers have reported that abnormal lipid metabolism is one of the major factors in the development of atherosclerosis. The progression of atherosclerotic lesions is accompanied by tissue lesions, which form plaques with lipid accumulation under the endothelial interstitium. Characteristics such as angiogenesis and plaque rupture can be described [3] [4]. This disruption leads to thrombus formation and ischemic lesions. Reperfusion is necessary as soon as possible, but the details of the factors affecting thrombolytic activity at this time are not clear. The effects of various food components on the development of atherosclerosis have been reported [5] [6] [7]. However, excessive intake of a particular component is dangerous [8] [9] [10]. On the other hand, it is difficult to believe that the intake of nutritionally balanced foods rich in various nutrients will have a negative effect on various mechanisms in the body. In this study, we examined the effects of chlorella intake as a food. Chlorella has long been consumed because it is rich in necessary nutrients such as high-quality protein, vitamins, and minerals [11]. In recent years, chlorella has attracted attention as a health food because of its excellent nutritional balance [12]. Chlorella has also been reported to exhibit physiological effects such as immune activation, growth promotion, and improvement of stress ulcers [13] [14] [15]. Since chlorella contains a variety of antioxidants and other nutrients [16] [17], it is speculated that chlorella-containing multicomponent supplements may be useful in reducing atherosclerosis. However, the effect of chlorella intake on atherosclerosis is unknown. To investigate the effect of chlorella on the clinical course of atherosclerosis, we investigated the onset and progression of athe-

rosclerosis, and thrombolysis after thrombus formation due to plaque disruption in ApoE$^{-/-}$ and Ldlr$^{-/-}$ double knockout mice. The purpose of this study was to experimentally evaluate the effects of chlorella on the development and progression of atherosclerosis and on thrombolytic activity after thrombus formation due to the disruption of the plaque.

2. Material & Methods

2.1. Experimental Animals, Chlorella Administration and Experimental Protocols

C57BL/6J × 129Sv double knockout male mice lacking both apolipoprotein E and the LDL receptor (B6; 129-ApoE tm1Unc Ldlr tm1Her/J mice, hereafter DK mice) were purchased from The Jackson Laboratories (Bar Harbor, Maine USA) and bred to siblings. C57BL/6J male mice were purchased from SLC Japan (Hamamatsu, Japan) and used in the experiments. Mice were kept in a chip cage (Softchip, Japan SLC Co., Ltd.) in the Kobe Gakuin University animal room (12 hours each of light and dark, room temperature 22.5˚C ± 0.5˚C, humidity 55% ± 5%). The animals were fed ad libitum with tap water and with either a standard solid diet CMF provided by Oriental Yeast Co.

The handling of experimental animals was in accordance with the "Basic Policy on Animal Experiments in the Field of Physiology" and the "Kobe Gakuin University Bioethics Policy on Research and Education" established by the Physiological Society of Japan. Chlorella was supplied by SUN CHLORELLA CORP (Kyoto, Japan).

The experimental diet consisted of standard solid feed CMF (Oriental Yeast Co., Ltd., Tokyo, Japan) to which Chlorella powder was added to prepare an experimental diet containing 1.65% Chlorella (equivalent to the standard human intake), and agar (final concentration 2%, Fujifilm Wako Pure Chemicals Co. Oosaka, Japan) and kept frozen in a −20˚C freezer until fed as experimental diets.

DK and C57BL/6J mice were pre-fed with standard solid diet (CMF) until 4 weeks of age, then divided into two groups, Chlorella-fed and Placebo-fed, for 18 weeks, and at 22 weeks of age, the degree of progression of arteriosclerosis, spontaneous thrombolytic capacity, blood samples, and blood vessel and liver samples were removed for various assays. Blood samples, blood vessels, and livers were removed and subjected to various assays.

2.2. Arteriosclerosis Evaluation Methods

1) Staining of atherosclerotic foci

The Entire aorta method [18] was used to measure atherosclerosis development. Mice anesthetized with Somnopentyl diluted 5-fold in saline was opened at the chest and abdomen, and a winged needle was placed in the left ventricle. After washing with phosphate buffer solution (PBS, pH 7.4), followed by reflux fixation in 10% neutral buffered formalin solution, the heart to the femoral artery was cut out. The subclavian artery and its branches to the right and left ca-

rotid arteries were then cut, and adipose and connective tissues attached to the vessels were carefully removed. The vessel lumen was inverted through a longitudinal incision. They were spread over a black rubber sheet using a 0.15 mm diameter stainless steel pin and stored in 10% neutral buffered formalin fixative until staining. They were washed with distilled water for 30 seconds and immersed in 60% isopropyl alcohol for 1 minute. The stained specimens were immersed in Oil Red O (Sigma-Aldrich Chemie GmbH, Germany) pre-warmed at 37˚C for 15 minutes, immersed in 60% isopropyl alcohol for 2 minutes, and then washed with distilled water for 2 to 3 minutes.

2) Analysis of atherosclerotic sites

Analysis of atherosclerotic areas was performed according to the method of T. Yamashita *et al.* [18]. The area of the area including the entire extracted blood vessel (W) and the area of the red area corresponding to the atherosclerotic area stained with Oil Red O stain (R) were determined using the image analysis software Image-Pro Puls (Media cyberics USA). Using these two area values, the ratio of the area of the atherosclerotic area to the area of the entire blood vessel (R divided by W) × 100 was calculated, and this was defined as the atherosclerotic lesion. The three-dimensional analysis of the atherosclerotic site layer was also analyzed from the surface brightness values using the same image analysis software.

2.3. *In Vivo* He-Ne Laser-Induced Thrombolysis Model

A polyethylene tube (PE-10; Becton Dickinson, Franklin Lakes, NJ) was placed in the left femoral artery of the mouse. The vessels on the mouse testicular elevator muscles were then exposed and secured with clips. Tyrode Buffer at 37˚C was dripped as needed to prevent desiccation. The operated mice were fixed on a microscope (Model BX; Olympus Co., Ltd., Tokyo, Japan) stage on a chamber heated to 37˚C. A 30 μm diameter vessel running over the cremaster muscle was selected, and a He-Ne laser (DPS-5002; NEOARK, Japan) with an output power of 20 mW was focused to 5 μm under the objective lens, irradiated for 5 seconds, and interrupted for 25 seconds. This was repeated to form a stable mural thrombus that stenosed 80% of the target vessel lumen.

2.4. Analysis of Thrombolysis Ratio over Time

After the stable mural thrombus that stenosed 80% of the vessel lumen had formed, the thrombus was video-recorded for 60 minutes using a DVD recorder (DVR-710H; Pioneer, Tokyo, Japan). The recorded thrombus images were captured on a PC at each time point (0, 10, 20, 30, 40, 50, and 60 minutes), and the optical integrated density was calculated as an approximation of thrombus size using Image-Pro Plus image analysis software (Media cybertics, Rockville, MD). After thrombus formation, the thrombus volume at minute 0 was set as 100%, the thrombus volume at minute 0 as the denominator, and the thrombus volume at each time point (10, 20, 30, 40, 50, and 60 minutes) as the numerator to calculate the thrombolytic ratio at each time point.

2.5. Gene Expression Analysis

After reflux washing in PBS, ascending aorta, descending aorta, liver, and kidney were collected, weighed, and flash frozen in liquid nitrogen. They were then stored frozen at −80˚C until mRNA extraction.

The mRNA was extracted from samples frozen and stored at −80˚C using RNeasy® Fibrous Tissue (Qiagen: Venlo, Nederland). Extracted mRNA was used to produce cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems: CA, USA) and stored frozen at −30˚C until use.

The concentration and purity of mRNA and cDNA were confirmed by measuring absorbance at A260 and A280 nm using a Thermo Scientific™ NanoDrop™ One Ultraviolet-Visible Spectrophotometer (Thermo Fisher Scientific: US-MA, USA).

The generated cDNAs were analyzed using TaqMan Fast Advanced Master Mix (Applied Biosystems: CA, USA) and TaqMan assay primers and probes (Applied Biosystems: CA, USA) in a Roche Real Time PCR was performed on a Roche LightCycler 96 (Roche Diagnostics K.K.: Tokyo, Japan). Results were processed using the comparative Ct method (ΔΔCt method) with β-actin as the endogenous control.

2.6. Statistical Processing

Statistical significance tests were performed using Paired t-test between two groups with correspondence, Student's t-test for equal variance between two groups without correspondence, and Mann-Whitney's U test for unequal variance between two groups without correspondence. The results are expressed as Mean ± SEM. Results are expressed as Mean ± SEM, with $P < 0.05$ indicating a significant difference.

3. Results

3.1. Body Weight Change

After 18 weeks of feeding the experimental diet in both groups, body weight at 22 weeks was 36.5 ± 0.975 g (n = 13) in the placebo group and 34.2 ± 0.603 g (n = 21) in the Chlorella group. No significant weight change was observed in the Chlorella group compared to the placebo group.

3.2. Comparison of Atherosclerosis Progression

Representative arteriograms and image analysis results for each group at 22 weeks of age are shown in **Figure 1**. The degree of atherosclerosis progression in each group was 20.4% ± 2.9% (n = 6) in the placebo group and 14.6% ± 3.4% (n = 6) in the Chlorella group. As seen in **Figure 1(B)**, there was no statistically significant difference in the area of atherosclerosis compared to placebo. However, the thickening of the atherosclerotic layer in the aortic arch and other atherosclerotic areas was clearly greater in the Placebo group than in the Chlorella group (**Figure 2**).

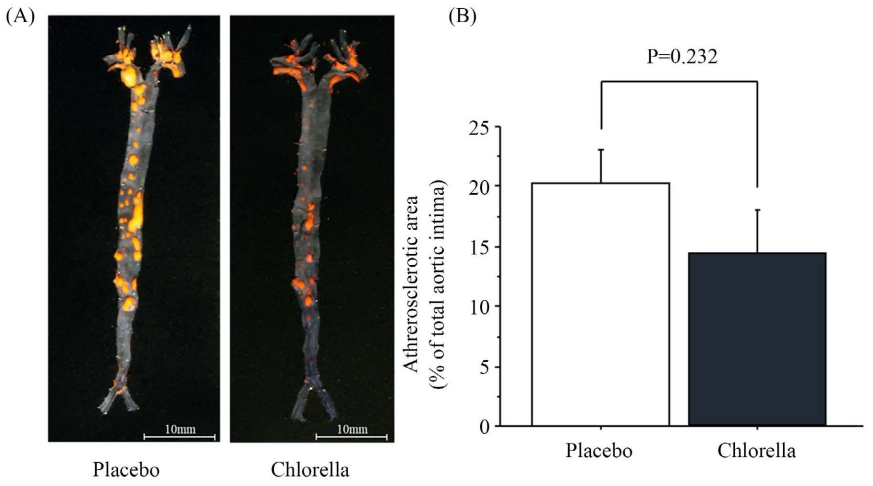

Figure 1. Comparison of atherosclerotic areas by Entire aorta method. (A) Vascular Oil Red O stained image (B) Comparison of atherosclerosis area by image analysis, n = 6/group.

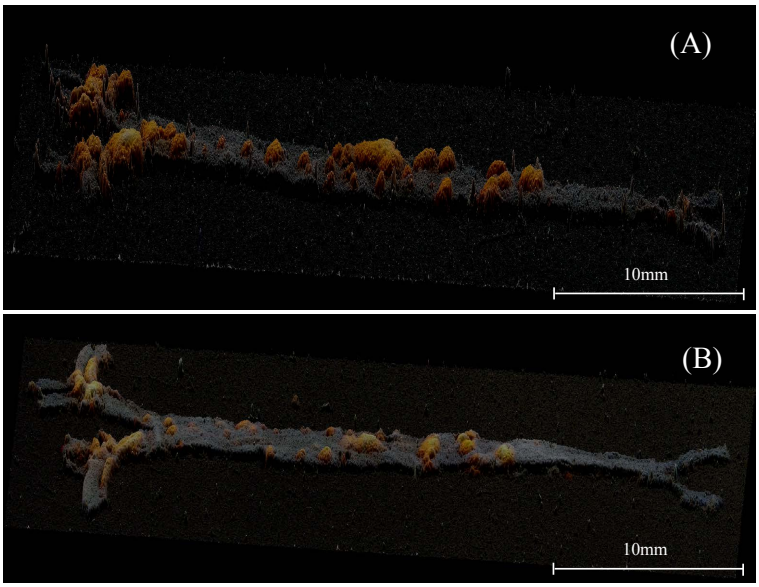

Figure 2. Representative example of comparison of thickened stereological analysis of atherosclerotic areas. (A) Placebo group, (B) Chlorella group.

3.3. Blood Lipid Levels and mRNA Levels of Factors Related to Metabolism

Serum TG levels (mg/dL) were 166.5 ± 41.7, 147.4 ± 22.6 (P = 0.696) for placebo and Chlorella groups, and serum T-Cho levels (mg/dL) were 804.8 ± 80.2, 739.2 ± 24.7 (P = 0.452) for placebo and Chlorella groups, Serum LDL-C (mg/dL) was 190.8 ± 22.1 and 184.9 ± 11.6 in the placebo and Chlorella groups, respectively (P = 0.817), Serum HDL-C (mg/dL) was 14.9 ± 2.2, 14.2 ± 1.0 (P = 0.777) in the placebo and Chlorella groups, showing no significant difference between the two groups, but a trend toward a decrease in serum TG, T-Cho and LDL-C was observed in the Chlorella group compared with the Placebo group.

The mRNA levels of SREBP-1, SREBP-2, Cyp7a1, FXR, LXR, as genes related to lipid metabolism were shown in Figure 3. mRNA levels of SREBP-1: 0.999 ± 0.11, 0.644 ± 0.06 (P = 0.0228) in placebo and Chlorella groups,

SREBP-2: 1.000 ± 0.143, 0.914 ± 0.152 (P = 0.386) for placebo and Chlorella, Cyp7a1: 0.467 ± 0.179, 0.1.589 ± 0.268 (P = 0.0087) for placebo and Chlorella, LXR Chlorella group were 1.001 ± 0.127, 1.468 ± 0.105 (P = 0.0035), SREBP-1 was significantly lower in the Chlorella group compared to the placebo group, and Cyp7a1 and LXR were significantly higher in the Chlorella group compared to the placebo group.

3.4. mRNA Levels of Inflammatory Markers

The mRNA expression levels of IL-6, MMP-9, MCP-1, and TNF-α as inflammatory markers associated with the development of atherosclerosis were compared between the placebo and Chlorella groups (Figure 4). IL-6: 0.999 ± 0.246, 0.436 ± 0.185 for placebo and Chlorella groups; MMP-9: 1.000 ± 0.196, 0.549 ± 0.159 for placebo and Chlorella groups; MCP-1: 0.999 ± 0.164. The values of MCP-1 and TNF-α in the placebo and Chlorella groups were 0.999 ± 0.164, 0.895 ± 0.286, and 1.000 ± 0.526, 0.895 ± 0.286, respectively. A trend toward decreased expression of IL-6 and MMP-9 was observed in the Chlorella group compared to the placebo group.

3.5. Thrombolysis over Time *in Vivo*

The thrombus volume ratio at stabilized thrombus formation and at 60 minutes was 71.6% ± 7.7% (n = 8) in the placebo group and 73.0% ± 7.9% (n = 7) in the Chlorella group; there was no statistically significant difference in the thrombus volume ratio at 60 minutes (Figure 5(B)), but the thrombus flattened compared to the placebo group (Figure 5(A)).

3.6. mRNA Levels of Fibrinolytic Markers

The mRNA levels of t-PA, which stimulates fibrinolysis, and PAI-1 and TAFI, which inhibit fibrinolysis, were compared between the placebo and Chlorella groups (Figure 6). t-PA: 1.001 ± 0.245, 0.855 ± 0.209 in the placebo group and the Chlorella group, PAI-1 and Chlorella group were 1.000 ± 0.318, 0.625 ± 0.070, and TAFI: placebo and Chlorella group were 1.000 ± 0.269, 0.552 ± 0.072. There was no difference in t-PA mRNA levels in the Chlorella group compared to the placebo group, but there was a decreasing trend in PAI-1 and TAFI, indicating a decreasing trend in the fibrinolytic inhibitory system.

4. Discussion

In this study, the effects of long-term Chlorella consumption on the development of atherosclerosis and thrombolytic potential were examined in an *In vivo* model. Double-knockout mice did not significantly suppress the progression of atherosclerosis. However, it significantly decreased SREBP-1 mRNA expression levels and increased LXR and CYP7a1 mRNA expression levels, which play a

central role in cholesterol catabolism in lipid metabolism involved in the development and progression of atherosclerosis. These results suggest that Chlorella may improve lipid metabolism by renewing cholesterol catabolism and enhancing bile acid synthesis.

Originally, in this experimental model, SREBP-1 expression was presumably enhanced by oxidized cholesterol induced from hypercholesterolemia, but Chlorella reduced its expression. Chlorella also increased the expression of CYP7a1, the rate-determing enzyme in the cholesterol catabolic pathway, and LXR, the rate-determing enzyme in bile acid synthesis. This suggests that enhanced CYP7a1 and LXR enhance bile acid synthesis. Thus, the results suggest that Chlorella induces suppression of SREBP-1 expression levels and enhancement of CYP7a1 and LXR expression levels, and improves lipid metabolism by increasing cholesterol catabolism.

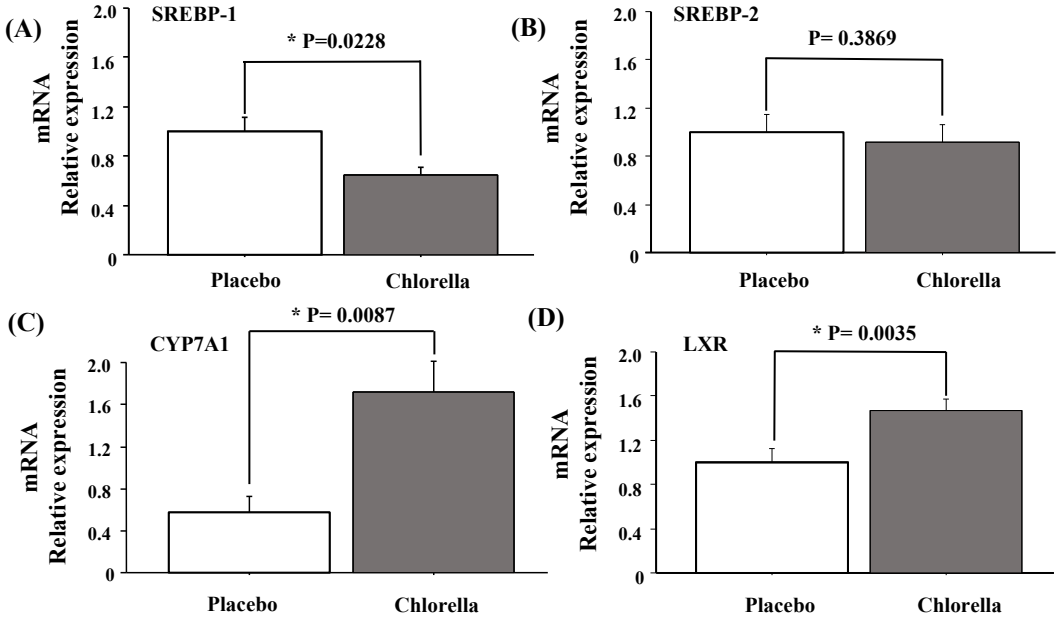

Figure 3. Comparison of mRNA expression levels of lipid metabolism mediators. (A) SREBP-1, (B) SREBP-2, (C) CYP7A1, (D) LXR Liver samples, n = 6 per group. *: P < 0.05.

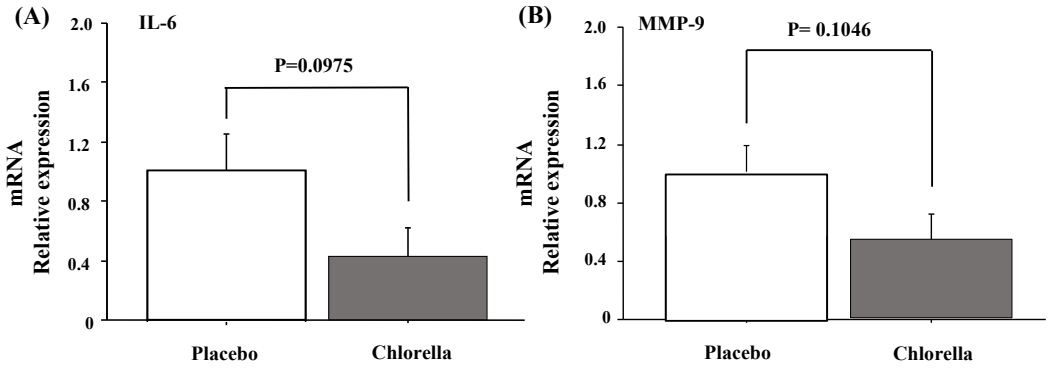

Figure 4. Comparison of inflammatory mediator mRNA expression levels. (A) IL-6, (B) MMP-9 Vascular samples, n = 6 per group.

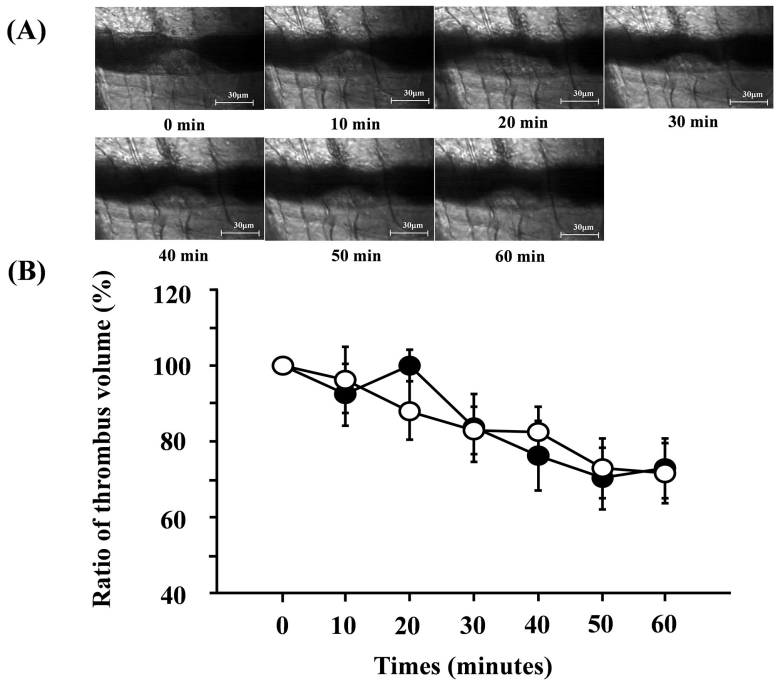

Figure 5. Thrombolysis ratio over time in both groups. (A) Thrombus image over time (B) Change in thrombus volume over time. ○: placebo group, ●: Chlorella group, n = 7 - 8/group.

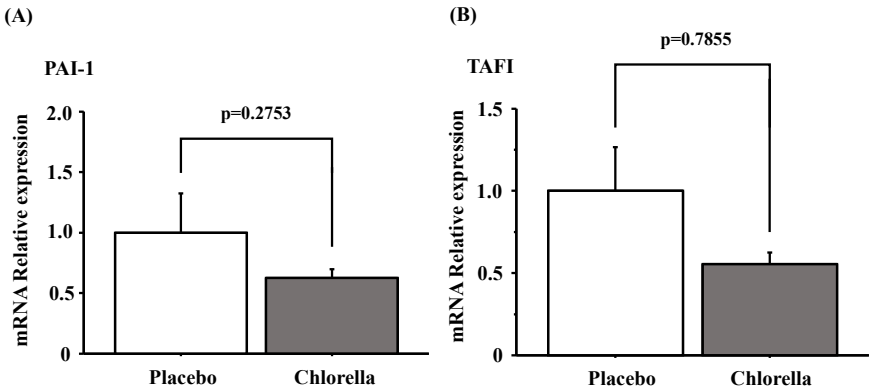

Figure 6. Comparison of mRNA levels of fibrinolysis inhibitors in the two groups. PAI-1 (Vascular samples) (B) TAFI (liver samples), n = 6 per group.

The double-knockout mice used in this study could not inhibit the development of significant atherosclerosis because they are double deficient in Ldlr and ApoE, which is considered to easily develop atherosclerosis, and in the method used to evaluate the atherosclerotic layer, In addition, the method used to evaluate the arterial stiffening layer was based on the area of the stiffening layer relative to the entire artery, but the stiffening layer in the Placebo group was thicker than that in the Chlorella group, even in the same stiffening layer, suggesting the need to change the evaluation method to volume. In addition, SREBP-2 has a role in suppressing the transcription of genes related to cholesterol metabolism in response to high cholesterol levels, whereas SREBP-1 does not. Therefore,

Chlorella does not affect SREBP-2 and does not interfere with the suppression of cholesterol production in hypercholesterolemia, which is also thought to have a positive effect on lipid improvement. In addition, the expression of IL-6, TNF-α, and MCP-1 as inflammatory markers, as well as MMP-9, egr-1, and VEGF-α gene expression, which are mediators involved in angiogenesis observed during the progression of atherosclerosis and VEGF-α gene expression levels were investigated. The expression of IL-6 mRNA and MMP-9 were suppressed in the control group. These results suggest that Chlorella, in combination with its ability to improve lipid metabolism, may induce an anti-inflammatory effect in atherosclerosis, an inflammatory disease.

Plaque disruption in atherosclerotic disease leads to severe pathology. The fibrinolytic kinetics of atherosclerosis are of interest because of the occlusive disease caused by thrombus formation, but the fibrinolytic kinetics associated with the development of atherosclerosis are not clear [19].

We previously reported that in ApoE$^{-/-}$ and LDLR$^{-/-}$ double knockout mice, long-term inhibition of thrombin markedly inhibited the development of atherosclerosis and enhanced spontaneous thrombolysis with decreased immunological expression of PAI-1 and TAFI [20]. In the present study, we investigated the effect of long-term consumption of chlorella as a food, rather than as a drug, on spontaneous fibrinolytic activity in the development of atherosclerosis. There are no reports examining the effects of chlorella used in this study on the blood coagulation cascade and the fibrinolytic cascade.

Long-term intake of chlorella did not show significant differences in this thrombolytic model.

Levels of t-PA mRNA, a fibrinolytic activator, also did not differ between the two groups. However, both PAI-1 and TAFI mRNA levels tended to decrease in the chlorella group compared to the placebo group, suggesting that chlorella intake may induce a decrease in fibrinolytic inhibitors mRNA levels. This paper is the first to report that chlorella consumption may induce a decrease in mRNA levels of fibrinolysis inhibitors. The surface expression of fibrinolysis is the result of a dynamic balance between activating and inhibitory effects. Therefore, the action of chlorella ingested as food to induce a decrease in PAI-1 and TAFI mRNA levels at normal doses is interesting. The mechanism is currently unknown, but may result in mild fibrinolytic activation. This experiment does not clarify what components of chlorella as a food product increase cholesterol catabolism, improve lipid metabolism, and decrease the expression of fibrinolytic inhibitory factors. Although further studies are needed, these results suggest that chlorella as a food may have positive effects on health maintenance.

Conclusion Long-term intake of chlorella may inhibit the progression of atherosclerosis by inducing increased cholesterol catabolism and bile acid synthesis and improving lipid metabolism in the clinical course of atherosclerosis, and may promote thrombolysis by inducing a decrease in mRNA levels of fibrinolytic inhibitory factors.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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