



Studies on Preventive Activity of Fucoxanthin and Fucoidan in Laminaria japonica from Life Style Related Diseases

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**Studies on Preventive Activity of Fucoxanthin and Fucoidan
in *Laminaria japonica* from Life Style Related Diseases**

ラミナリアジャポニカに含まれるフコキサンチンとフコイダンの
生活習慣病予防活性に関する研究

Rendong Ren

任 人棟

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Abbreviations

CVDs	cardiovascular diseases
DAPI	4'6-diamino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dTxB ₂	11-dehydro-thromboxane B ₂
<i>DUOX2</i>	dual oxidase 2
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FD	fucoidan group
FX+FD	fucoidan+fucoxanthin group
FX	fucoxanthin group
GTT	global thrombosis test; OT, occlusion time
HPLC	high performance liquid chromatography
H ₂ O ₂	hydrogen peroxide
6-keto-PGF _{1α}	6-keto-prostaglandin F _{1α}
ICR	imprinting control region
Ig	immunoglobulin
IL	interleukin
LDL	low density lipoprotein
LT	lysis time
α-MEM	α-minimal essential medium
NF-κB	nuclear factor-κB
<i>NOX1</i>	NAD(P)H oxidase 1
9-ONA	9-oxononanoic acid
PAGE	polyacrylamide gel electrophoresis
PBS,	phosphate-buffered saline
PGI ₂	prostacyclin
PLA ₂	phospholipase A ₂
PI	propidium iodide

PUFAs	polyunsaturated fatty acids
RANKL	receptor activator of nuclear factor κ B ligand
RNA	ribonucleic acid
RNase A	ribonuclease A
SDS	sodium dodecyl sulfate
TRAP	tartrate-resistant acid phosphatase
TxA ₂	thromboxane A ₂
TxB ₂	thromboxane B ₂
UV	ultraviolet

Chapter 1

General introduction

Diet and life style are major factors which influence many diseases. Some unhealthy life styles such as drug abuse, tobacco smoking, and alcohol drinking increase the risk of developing certain diseases, especially later in life. The life style related diseases mainly include cancer, osteoporosis and atherosclerosis et al.

It has been reported cancer incidence is closely associated with the life style [1]. in the 1970s, people in many western countries had diets high in animal products, fat, and sugar, and high rates of cancers of the breast, prostate, and lung; by contrast, individuals in developing countries usually had diets that were based on one or two starchy staple foods, with low intakes of animal products, fat, and sugar, and low rates of these cancers [2]. Not only cancers, but also osteoporosis is associated with the life style. Osteoporosis is a disease of bones which leads to an increased risk of fracture. In osteoporosis the bone mineral density is reduced, epidemiologic evidence suggests that life style factors, such as exercise, excessive alcohol intake, and tobacco consumption, are associated with bone mineral density [3-5]. Hip fracture is the most severe consequence of osteoporosis, leading to reduced activities of daily living, lowered quality of life, and increased mortality of patients [6, 7]. Osteoporosis is estimated to affect 200 million women worldwide - approximately one-tenth of women aged 60, one-fifth of women aged 70, two-fifths of women aged 80 [8]. As the population ages in Japan, the total number of hip fractures is forecast to be 238,000 per year in 2030 [9]. Therefore, osteoporosis has become a serious threat to society.

In addition to cancer and osteoporosis, life style is also influence the cause of atherosclerosis. The major manifestations of atherosclerosis are coronary artery disease (myocardial infarction, angina, sudden death), cerebrovascular disease (ischemic stroke), and peripheral vascular disease (ischemic limbs), all of these diseases are referred as cardiovascular diseases (CVDs). It has been reported atherosclerosis is the leading underlying cause of mortality in industrialized countries

in the last two decades [10]. So far, the situation is still serious. According to the report from American Heart Association, more than 81 million Americans suffer from some form of cardiovascular disease, making it the leading cause of death in the country. As of 2006, cardiovascular disease was responsible for at least one in every 2.9 deaths in the United States. CVD claims more lives each year than cancer, chronic lower respiratory disease, and accidents combined [11].

Preventing of these diseases is an urgent task to society. Many human epidemiological studies and animal studies showed that high intake of fruits and vegetables over a prolonged period of time reduced cancer disease [12-14]. Thus, cancer preventive plant has been attracted a great attention and the plant is also expected to have effect on osteoporosis and CVD. Various factors in plant food, such as antioxidative vitamin, carotenoids, phenolic, terpenoid and fibers, have been considered as responsible for reducing the life style related diseases.

Seaweeds are traditional diet of East Asians particularly in Japan which is a source of glutamic acid, dietary fibers, minerals and vitamins. Japanese traditionally eat a large amount of seaweed and the life expectancy is the highest in the world [15]. Japanese kelp, namely kombu (*Laminaria japonica*) is one of the popular seaweeds and which have been consumed 133,000 ton in fresh weight per year [16]. It has been reported that kombu prevents either the initiation of breast cancer or its promotion by endogenous physiological factors [17]. Kombu includes xanthophyll-fucoanthin and zeaxanthin, phenolics-protocatechuic acid, polysaccharide fucoidan, dietary fiber β -glucan, and chlorophyll. Among them, two particular compounds, fucoidan and fucoxanthin, they has been attracted great attention and studied extensively recent years. Fucoidan is a polysulfated polysaccharide found primarily in various species of brown seaweed. Fucoidan has been reported to have various biological effects such as antitumor activity by enhancing the immune response of hosts [18], the anti-allergy activity by preventing the NF- κ B p52-mediated pathways through CD40 [19], and anti-thrombosis activity in cells and animal experiments [20-22]. It also reports that fucoidan therapy decreases the proviral load in patients with human T-lymphotropic

virus type-1-associated neurological disease [23]. Moreover, study has indicated that fucoidan did not show toxicity when rats were administered with fucoidan as much as 300 mg/kg of body weight per day [24]. Thus, it's promising to develop kelp which containing fucoidan as a functional food to prevent from life style related disease.

The other interesting compound in kelp is fucoxanthin. Fucoxanthin is an oxygen-containing carotenoid xanthophyll with a unique allene linkage, and it is incorporated into the bloodstream after undergoing hydrolysis of the side-chain acetyl group to fucoxanthinol when ingested [25]. Fucoxanthin has been reported to have potent beneficial effects on human health. Fucoxanthin is showing preventing colon adenoma through the induction of cell cycle arrest by up-regulation of p21^{WAF1/Cip} [26], preventing liver [27] and skin [28] cancer due to its antioxidant activity, preventing breast and colon cancer through the induction of apoptosis in cancer cells [29], anti-obesity effects by stimulating the expression of uncoupling protein 1 in white adipose tissue [30, 31]. The anti-carcinogenic activity of fucoxanthin is reported to be the strongest among xanthophylls and carotenoid [28]. In addition, anti-angiogenic effect [32] and anti-inflammatory activity [33] of fucoxanthin are also reported. Moreover, fucoxanthin is no side effect even with an extremely high intake: as much as 0.27% fucoxanthin diet on mice, equivalent to around 0.25 mg/kg body weight/day for 4 weeks, mice didn't show any abnormalities [30]. Epidemiological studies have provided evidence that plant-derived diets can reduce the risk for cardiovascular disease and stroke [34]. However, whether fucoidan and fucoxanthin have preventive effects on osteoporosis and cardiovascular diseases or not has been never reported before.

In this study, the author investigated the preventive effect of fucoidan and fucoxanthin extracted from Japanese kelp, on osteoporosis and atherosclerosis and tried to clarify the mechanism. In chapter 2, the author found that fucoxanthin significantly inhibited the survival of osteoclasts differentiated from RAW264.7 cells through inducing apoptosis by an activation of caspase-3, but did not affect on survival of pre-osteoblastic MC3T3 E1 cells. Thus, fucoxanthin seems likely to be

preventive from bone-resorption related-diseases such as osteoporosis and rheumatoid arthritis. In chapter 3, the author found dietary fucoidan acted on intestinal epithelial cells to release signal, H_2O_2 , which modulated eicosanoid secretion resulting in a shortened lysis time for thrombi in humans. Thus, intake of dietary fucoidan seems to have anti-thrombosis effect. In chapter 4, the author extended the study to investigate the mechanism that development of atherosclerosis. The author found that 9-ONA worked as a signaling chemical to induce PLA_2 activity and activate arachidonate cascade which promoted the progression of atherosclerosis. Finally, in chapter 5 the beneficial effect of fucoxanthin and fucoidan on human health is discussed.

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Chapter 2

Fucoxanthin induces apoptosis in osteoclast-like cells differentiated from RAW264.7 cells

2.1 Introduction

Osteoclasts, a large multinucleated cell generally resorbs bone, maintain calcium homeostasis, and support normal bone remodeling [1]. However, excess osteoclast activity leads to an imbalance in bone remodeling, which favors resorption [2], and induces osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma, and metastatic cancers [3]. Osteoclasts are formed through the fusion of mononuclear precursors by a multistep differentiation process under the control of the bone microenvironment, which includes stromal cells, osteoblasts, and local factors [3]. In vitro studies found that at least two extracellular factors, macrophage-colony stimulating factor and receptor activator of NF- κ B ligand (RANKL), are implicated in the differentiation of osteoblast to osteoclast [4-8]. Other factors, such as interleukin (IL)-1, IL-6, tumor necrosis factor γ , and transforming growth factor β , also affect osteoclast differentiation. It is feasible to use in vitro models to modulate osteoclast differentiation and predict the effects on bone resorption in vivo and RAW264.7 cells in the presence of RANKL differentiate readily into pure populations of osteoclast-like cells [9]. These multinucleated cells express known osteoclast markers, and exhibit the capacity to form resorption pits on mineralized surfaces. As precursor cells differentiate and fuse to form mature osteoclasts, signature phenotype markers are expressed, including tartrate-resistant acid phosphatase (TRAP) [1, 10].

Fucoxanthin is an oxygenated carotenoid produced specifically by edible brown sea algae such as kombu (*Laminaria japonica*), wakame (*Undaria pinnatifida*) and arame (*Eisenia bicyclis*) has received considerable attention due to its scientifically proven beneficial effects on human health; suppressive effect on adipocyte differentiation [11], antimutagenicity [12], antiocular inflammation [13], and

preventive effects on colon, liver and lung cancers [14-17]. However, no studies have been published about the effects of fucoxanthin on bone health, although a number of studies on this subject have investigated the effects of other carotenoids. Lycopene, which is abundant in tomato, exhibits inhibitory effects on osteoclasts formation and resorption [18, 19] as well as stimulatory effects on cell proliferation and alkaline phosphatase, a differentiation marker of osteoblasts [20, 21]. β -Cryptoxanthin, which is abundant in citrus fruits, shows anabolic effects on bone calcification [22], stimulates bone formation and inhibits bone resorption in cultured bone tissues in vitro [23]. In rats with streptozotocin-induced diabetes and in ovariectomized rats, β -Cryptoxanthin also prevented bone loss [24] and had anabolic effects on bone components [25, 26]. We predict that fucoxanthin also possesses anti-osteoporotic effects.

In the present study, we examined the effects of fucoxanthin on the osteoclast differentiation of precursor RAW264.7 monocytes and its cytotoxicity against osteoclast-like cells differentiated from RAW264.7 cells and cells of the osteoblast-like cells line MC3T3-E1.

2.2 Materials and methods

2.2.1 Chemicals.

Propidium iodide (PI), ribonuclease A (RNase A), proteinase K, 4',6-diamino-2-phenylindole (DAPI), and recombinant mouse soluble receptor activator of nuclear factor κ B ligand (RANKL) were purchased from Sigma Chemicals (St. Louis, MO). For the cell culture, Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical, α -minimal essential medium (α -MEM) was from Wako Pure Chemical Industries (Osaka, Japan), and fetal bovine serum (FBS) was from Sigma Chemicals.

2.2.2 Preparation of fucoxanthin.

The fucoxanthin used in this study was isolated from the brown sea algae

Laminaria japonica (kombu), which was cultured and harvested at Minamikayabe in Hakodate city, Hokkaido Japan as described previously [27]. The purity of the fucoxanthin used in this study was more than 95% as determined by HPLC. Fucoxanthin was dissolved in dimethyl sulfoxide (DMSO) at 10 mM as the stock solution.

2.2.3 Cell culture.

Cell of the murine macrophage cell line RAW264.7 and cells of the osteoblast-like cell line MC3T3-E1 were maintained in DMEM containing 10% heat-inactivated FBS, 4 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere of 95% air-5% CO₂ at 37 °C.

2.2.4 Determination of cell viability.

Cell viability was determined using the trypan blue dye exclusion assay. RAW264.7 cells (2×10^3) cells were seeded onto 60 mm dishes, incubated for 12 h for adherence, and treated with fucoxanthin for 4 days. The cells were harvested by trypsinization, and stained with 0.4% (w/v) trypan blue (Gibco, Grand Island, NY) in phosphate-buffered saline (PBS). The trypan blue-unstained cells were counted using a hemocytometer under a phase-contrast microscope as visible cells. MC3T3-E1 cells (1×10^6) were seeded onto 100 mm dishes, and cell viability was determined according to the same method as used for the RAW264.7 cells.

2.2.5 Osteoclast differentiation.

RAW264.7 cells were seeded at a density of 6×10^3 cells/mL in α -MEM containing 10% FBS, that is 6×10^2 cells in a 96-well plate, 6×10^3 cells in a 12-wells plate, and 6×10^4 cells in a 100 mm dish. After the cells adhered, the medium was replaced with fresh medium, and the cells were treated with 50 ng/mL of recombinant mouse soluble RANKL and cultured for 4 days to induce osteoclast differentiation. TRAP expression, a phenotype biomarker of osteoclasts, was determined using an acid phosphatase-staining kit (TRACP & ALP double-stain Kit, Takara Bio, Shiga, Japan) according to the manufacture's protocol. The number of TRAP-positive multinucleated osteoclasts containing more than three nuclei was counted using a

phase-contrast microscope as an index of mature osteoclast-like cells that had differentiated from RAW264.7 cells.

2.2.6 Apoptosis analyses

In general, the apoptotic cells initially shrunk and then fragmented to form small apoptotic bodies [28]. Apoptosis analyses were performed by morphological observation and the detection of deoxyribonucleic acid (DNA) fragmentation in osteoclast-like cells differentiated from RAW264.7 cells.

For the morphological observation, the cells were washed with PBS and fixed with 70% ice-cold ethanol for at least 2 h at -20°C . The fixed cells were then washed with PBS and incubated with 1 mg/mL RNase A and 50 $\mu\text{g/mL}$ PI in PBS at room temperature for 30 min in the dark. The PI-stained nuclei were observed under a fluorescent microscope.

DNA fragmentation was measured as described previously [29]. Briefly, the cells in the 12-well plate were lysed with 200 μL of TE buffer [10mM Tris-HCl (pH8.0) and 1 mM ethylenediamineretraacetic acid] containing 0.5% Triton X-100, and the lysates were centrifuged at 17000g for 20 min at 4°C to separate fragmented from intact DNA. The supernatant containing fragmented DNA was transferred to another microtube, and the pellet containing intact DNA was dissolved in 200 μL of TE buffer containing 0.5% SDS. Both fractions were treated with 0.25 mg/mL RNase A at 50°C for 10 min and then with 0.25 mg/mL proteinase K at 50°C for 30 min. DNA was precipitated with 42 mM NaCl and 50% isopropanol for 6 h at -20°C , obtained by centrifugation at 17000g for 20 min, and labeled with 1 $\mu\text{g/mL}$ of DAPI in Tris-NaCl buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM EDTA] for 30 min. The fluorescent intensity was measured at 350 nm wavelength for excitation and at 452 nm for emission. DNA fragmentation was expressed as a percentage of fragmented DNA in the sum of intact and fragmented DNA.

For agarose gel electrophoresis, the fragmented DNA extracted by the precipitation with 50% isopropanol as described above was washed with ice-cold 70% ethanol and dissolved in 10 μL of TE buffer. The solution was applied to 2% agarose

gel electrophoresis, and fragmented DNA was visualized by ethidium bromide staining with an UV transilluminator.

2.2.7 Western blotting analysis.

Cells in the 100 mm dish were washed with PBS and lysed with RIPA buffer containing 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholic acid, and 0.05% SDS. The protein concentration of the cell lysate was measured according to the Lowry method [30]. Aliquots of 50 μ g were subjected to SDS-PAGE and transferred onto poly (vinylidene difluoride) membranes (Amersham Biosciences, Piscataway, NJ). The sources of the primary antibodies used in this study were as follows: those for cleaved caspase-3 (Asp175), caspase-3, and PARP were from Cell Signaling Technology (Beverly, MA), and that for β -actin was from Santa Cruz Biotechnology (Santa Cruz, CA). As a secondary antibody, horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Amersham Biosciences and Nacalai Tesque (Kyoto, Japan), respectively. The immunocomplexes were detected by enhanced chemiluminescence using the ECL Plus Western Blotting System (Amersham Biosciences).

2.2.8 Measurement of fucoxanthin and fucoxanthinol in Osteoclast-like Cells.

Amounts of fucoxanthinol and fucoxanthinol in osteoclast-like cells were measured as described previously [31] with a slight modification. Briefly, osteoclast-like cells in 12-well plates were lysed with 1 mL of TE buffer containing 0.5% Triton X-100, and the lysates were centrifuged at 17000g for 10 min at 4 °C. The supernatant was mixed with the same volume of ethyl acetate. The ethyl acetate layer was collected and dried by nitrogen gas. The residue was dissolved in 200 μ L of dichloromethane/methanol (1:4, v/v), and 20 μ L of the solution was subjected to HPLC analysis. The HPLC system equipped with an L-7420 detector was employed. The column (250 mm $\times$ 4.6 mm internal diameter) and a guard column (10 $\times$ 4.0 mm internal diameter) were of the Capcell pak C18 UG80 (Shiseido, Tokyo, Japan) and were maintained at 35 °C. The solvent used was acetonitrile/methanol/water (75:15:10, v/v/v) containing 1 mg/mL ammonium acetate, and the flow rate was 1.0 mL/min. Fucoxanthin and fucoxanthinol were monitored at a wavelength of 450 nm and identified by retention time by comparison with standards of fucoxanthin and

fucoxanthinol. These amounts were quantified from their peak areas by their standard curves.

2.2.9 Statistical Analysis.

The data are reported as the mean $\pm$ standard deviation (SD) of the values from three independent determinations. Statistical analysis was performed using Student's *t* test. Probability values less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Effect of fucoxanthin on viability of RAW264.7 cells.

The cytotoxicity of fucoxanthin on RAW264.7 cells were examined by the trypan blue exclusion assay (Fig. 1). RAW264.7 cells were cultured for 4 days in media containing various concentrations of fucoxanthin. At concentrations of $< 5 \mu\text{mol/L}$, fucoxanthin did not have detrimental effects on the survival of cells. However, $10 \mu\text{mol/L}$ fucoxanthin caused a significant decrease in the number of viable cells compared to vehicle alone (0.1% DMSO). The effects of fucoxanthin on the osteoclastogenesis of RAW264.7 cells were evaluated at concentrations of $< 5 \mu\text{mol/L}$ in the following experiments.

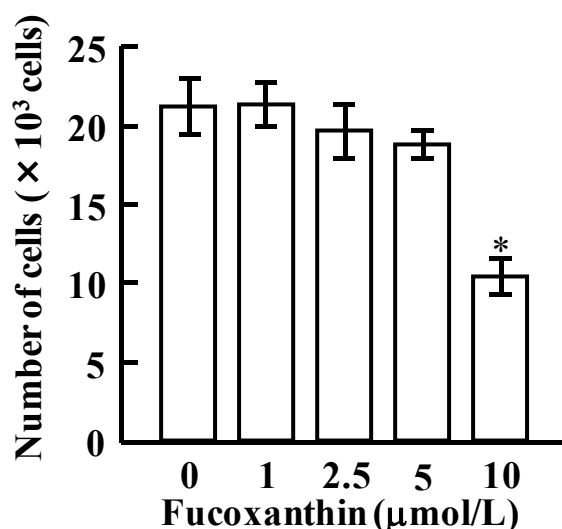


Fig. 1. Effect of fucoxanthin on the viability of RAW264.7 cells. Cells were treated with the indicated concentrations of fucoxanthin for 4 days. The viable cells were evaluated by the trypan blue exclusion assay. The data are representative of three independent experiments and are shown as the mean $\pm$ SD of the three experiments ($n = 6$ culture dishes); *, $p < 0.05$ versus fucoxanthin-untreated cells ($0 \mu\text{mol/L}$).

2.3.2 Suppressive effects of fucoxanthin on RANKL-induced osteoclastogenesis.

The treatment of RAW264.7 cells with RANKL (50 ng/mL) for 4 days increased

the number of TRAP-positive and multinucleated cells compared with control cells untreated with RANKL (Fig. 2). Therefore, the effects of fucoxanthin on the osteoclastogenesis of RAW264.7 cells were evaluated after treatment with 50 ng/mL RANKL for 4 days. After treatment with RANKL in the absence or presence of fucoxanthin, the TRAP-positive multinucleated cells were counted as osteoclast-like cells. Fucoxanthin at concentrations of $> 2.5 \mu\text{mol/L}$ caused a significant decrease in the number of osteoclast-like cells (Fig. 3A), suggesting that fucoxanthin inhibited the differentiation of RAW264.7 cells to osteoclast-like cells and/or that fucoxanthin induced cell death in the osteoclast-like cells. To investigate the cytotoxicity of fucoxanthin to osteoclast-like cells, osteoclast-like cells differentiated by RANKL stimulation were further cultured in the absence or presence of fucoxanthin (Fig. 3B).

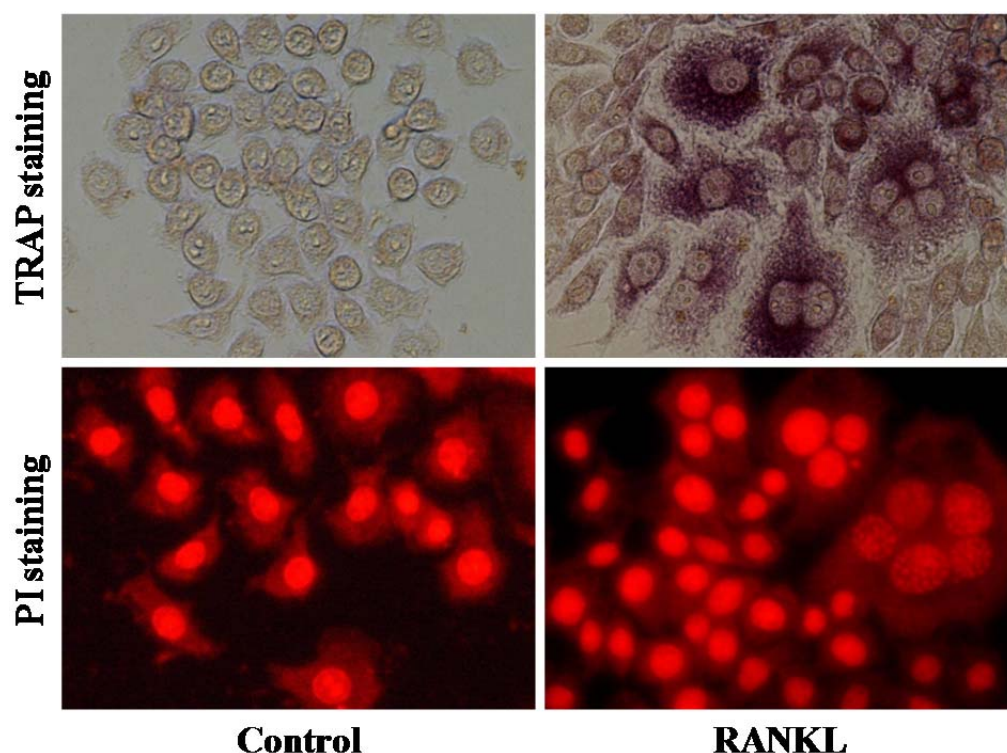


Fig. 2. Differentiation of RAW264.7 cells into osteoclast-like cells by RANKL. RAW264.7 cells were cultured with receptor activator of nuclear factor κB ligand (RANKL) or not (control) for 4 days and stained with a tartrate-resistant acid phosphatase (TRAP) staining kit or propidium iodide (PI). Osteoclast-like cells were observed as TRAP-positive and multi-nucleated cells.

Fucoxanthin treatment significantly decreased the number of cells by 24 and 48 h after treatment. Therefore, the decreases in the number of osteoclast-like cells shown

in Figure 3A were due to the cytotoxic effect of fucoxanthin on differentiating and/or differentiated cells.

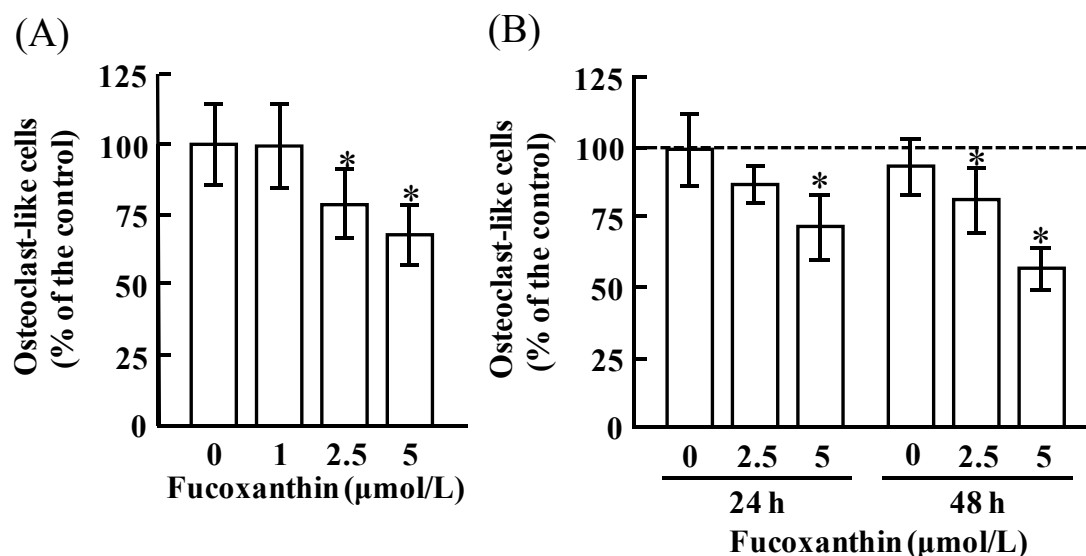


Fig. 3. Inhibitory effect of fucoxanthin on the osteoclast differentiation of RAW264.7 cells and the cell viability of osteoclast-like cells: (A) RAW264.7 cells were cultured with RANKL and the indicated concentrations of fucoxanthin for 4 days. The number of osteoclast-like cells was counted under a phase-contrast microscope following TRAP staining. The data are representative of three independent experiments and expressed as the mean $\pm$ SD of the three experiments ($n = 5$ culture dishes); *, $p < 0.05$ versus fucoxanthin-untreated control cells (0 $\mu\text{mol/L}$). (B) RAW264.7 cells were cultured with RANKL for 4 days and then cultured with the indicated concentrations of fucoxanthin for another 24 or 48 h. The number of osteoclast-like cells was counted under a phase-contrast microscope following TRAP staining. The values are expressed as the percentage of the cell number before treatment with fucoxanthin. The data are representative of three independent experiments and expressed as the mean $\pm$ SD of the three experiments ($n = 5$ culture dishes); *, $p < 0.05$ versus the values before treatment with fucoxanthin (100%).

2.3.3 Apoptosis induction by fucoxanthin in osteoclast-like cells.

To further investigate the cytotoxicity of fucoxanthin to osteoclast-like cells, osteoclast-like cells were treated with fucoxanthin for 48 h and morphologically observed after PI staining. Treatment with fucoxanthin at $> 2.5 \mu\text{mol/L}$ caused cell and nucleus shrinkage, nuclear fragmentation, and the formation of apoptotic bodies, which are characteristic of apoptotic cells (Fig. 4A). In addition to 48 h treatment, 24 h treatment with fucoxanthin at 5 $\mu\text{mol/L}$ also induced morphological changes but did not do so at 2.5 $\mu\text{mol/L}$ (data not shown). DNA fragmentation is another hallmark of apoptotic cells [28]. Fucoxanthin increased DNA fragmentation in a dose-dependent

manner 48 h after the treatment (Fig. 4B), and the agarose gel electrophoresis clearly showed the DNA ladder pattern characteristic of apoptosis by the treatment with 2.5 and 5 $\mu\text{mol/L}$ fucoxanthin (Fig. 4C). These results indicate that the cytotoxicity of fucoxanthin in osteoclast-like cells is accompanied by apoptosis induction.

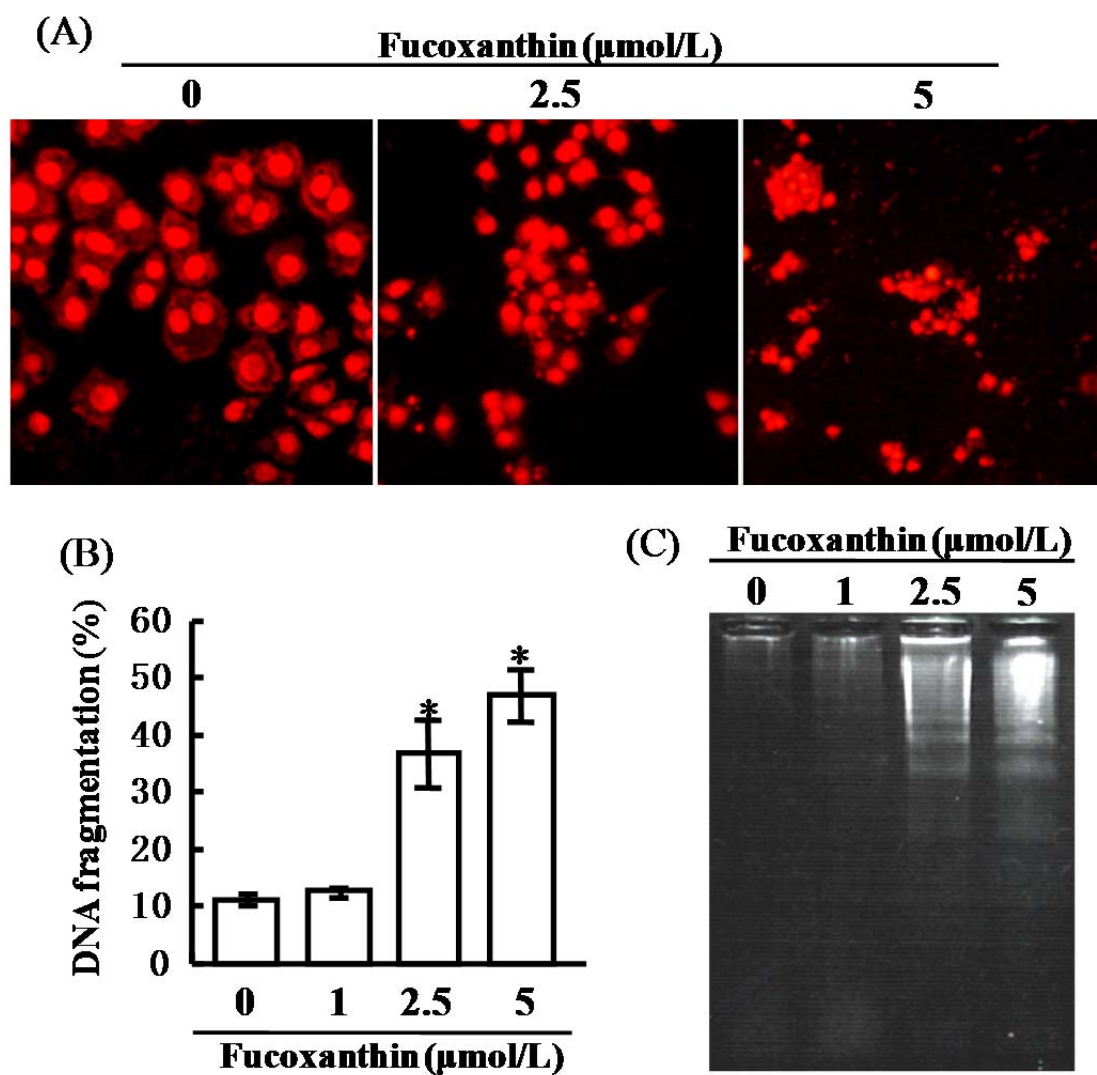


Fig. 4. Induction of apoptosis by fucoxanthin in osteoclast-like cells. Osteoclast-like cells were treated with the indicated concentrations of fucoxanthin for 48 h: (A) the morphological changes that occurred following PI staining were observed under a fluorescent microscope; (B) DNA fragmentation was quantitatively measured using DAPI {values were calculated as a percentage of fragmented DNA in total; data are represented as means $\pm$ SD [$n = 3$ culture dishes; *, $p < 0.05$ versus fucoxanthin-untreated control cells (0 $\mu\text{mol/L}$)]}; (C) nucleosomally fragmented DNA was analyzed by 2% agarose gel electrophoresis (data are representative of triplicate experiments).

2.3.4 Activation of caspase-3 in fucoxanthin-treated osteoclast-like cells.

To investigate the mechanism of fucoxanthin-induced apoptosis, the involvement

of caspase activation in the apoptosis of osteoclast-like cells was examined at 24 and 48 h after treatment with fucoxanthin at 2.5 or 5 $\mu\text{mol/L}$. Western blotting analysis showed that fucoxanthin treatment significantly increased the amount of the cleaved 17-kDa fragment of caspase-3, the active form, and decreased the amount of the 35 kDa procaspase-3 at 48 h, indicating that fucoxanthin activated caspase-3 (Fig. 5). In addition, fucoxanthin also caused the cleavage of PARP, an intracellular substrate of caspase-3. These results indicate that fucoxanthin activated caspase-3 and sequentially cleaved PARP, resulting in apoptotic induction.

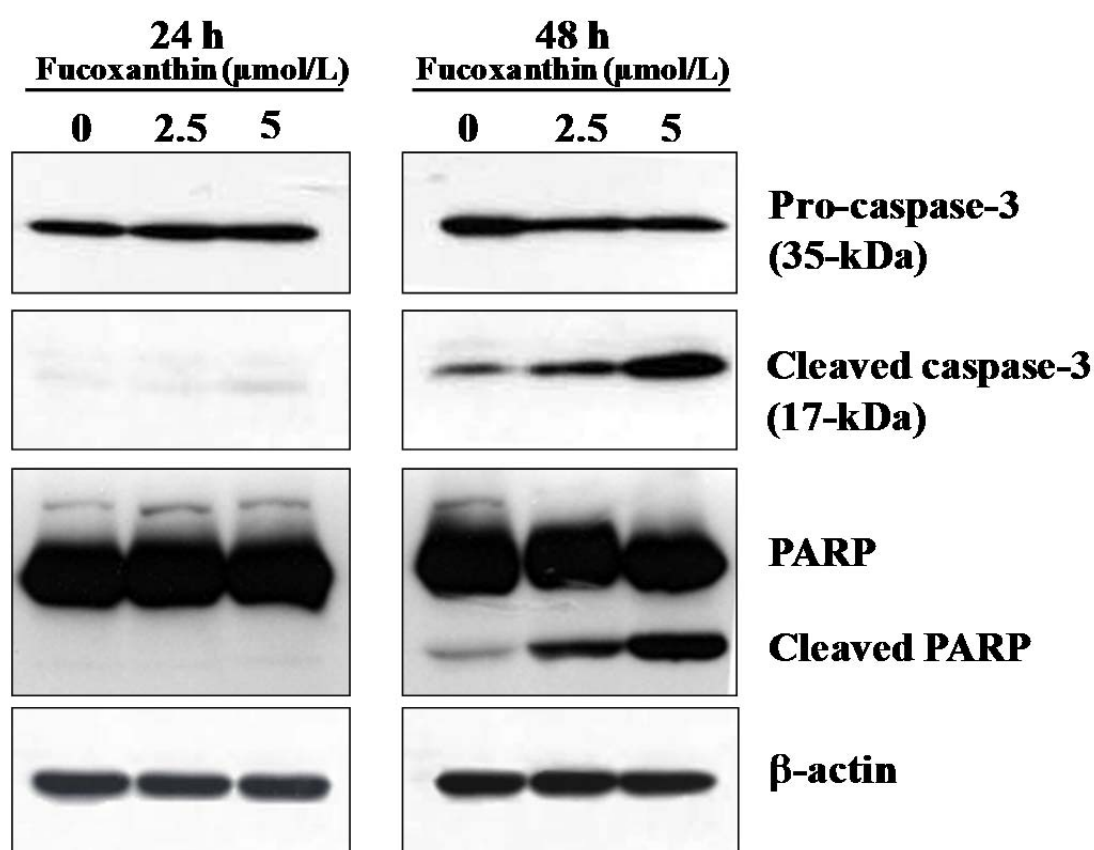


Fig. 5. Involvement of caspase-3 activation in fucoxanthin-induced apoptosis of osteoclast-like cells. Osteoclast-like cells were treated with the indicated concentrations of fucoxanthin for 24 or 48 h, and apoptosis-related proteins were analyzed by western blotting analysis. Equal loading of protein was confirmed by β -actin. The data are representative of duplicate experiments.

2.3.5 Cytotoxicity of fucoxanthin toward osteoblast MC3T3-E1 cells.

Bone remodeling is continually controlled according to physiological circumstances through bone resorption by osteoclasts and bone formation by osteoblasts [1-3]. To investigate the cytotoxic effects of fucoxanthin on osteoblasts,

cells of the osteoblast-like cell line MC3T3-E1 were cultured in several concentrations of fucoxanthin for 4 days (Fig. 6), and the effects were evaluated according to the same method as shown in Figure 3A. Fucoxanthin at 5 $\mu\text{mol/L}$ showed significant cytotoxicity, but did not at concentrations of $< 2.5 \mu\text{mol/L}$. These results indicate that the cytotoxicity of fucoxanthin against osteoclasts is stronger than that against osteoblasts.

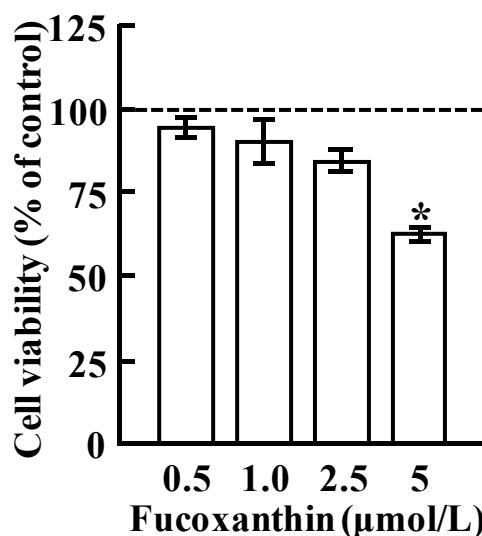


Fig. 6. Effect of fucoxanthin on the viability of MC3T3-E1 cells. MC3T3-E1 cells were treated with the indicated concentrations of fucoxanthin for 48 h, and the viable cells were counted by the trypan blue exclusion assay. The values are expressed as the percentage of fucoxanthin-untreated control cells, and the data represent the mean $\pm$ SD of three culture dishes; *, $p < 0.05$ versus fucoxanthin-untreated control cells.

2.3.6 Amount of fucoxanthin and fucoxanthinol in osteoclast-like Cells.

To investigate the intracellular concentrations of fucoxanthin and its metabolite fucoxanthinol, osteoclast-like cells were treated with fucoxanthin for 48 h, and the intracellular concentration was analyzed with HPLC. The major peaks completely fitted with the peaks corresponding to the standards for fucoxanthin and fucoxanthinol when the extract mixed with these standards was injected to the HPLC (data not shown). The intracellular fucoxanthin and fucoxanthinol increased in a dose-dependent manner (Fig. 7). More than 60% of fucoxanthin incorporated into cells was hydrolyzed to fucoxanthinol. This result indicates that fucoxanthinol may play an important role in inducing apoptosis in osteoclast-like cells.

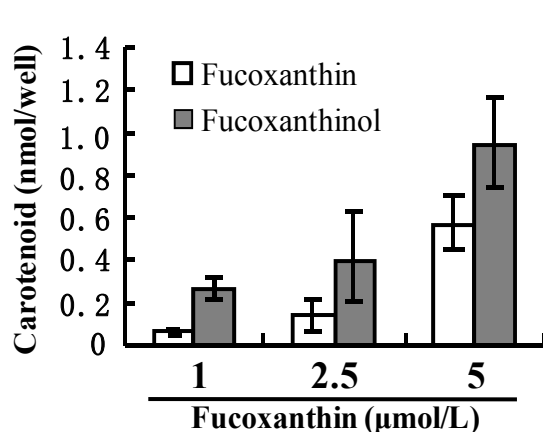


Fig. 7. Intracellular fucoxanthin and fucoxanthinol in osteoclast-like cells. Osteoclast-like cells were treated with 1 μmol/L (1 nmol/well), 2.5 μmol/L (2.5 nmol/well), or 5 μmol/L (5 nmol/well) fucoxanthin for 48 h. Cells were harvested, and intracellular fucoxanthin (open bars) and fucoxanthinol (solid bars) were extracted and analyzed by HPLC. The data represent the mean ± SD of three wells.

2.4 Discussion

In the present study, the effects of fucoxanthin on osteoclastogenesis were investigated using cells of the macrophage cell line RAW264.7, which have the capacity to differentiate into osteoclast-like cells after RANKL stimulation. Fucoxanthin significantly suppressed the differentiation of RAW264.7 cells at 2.5 μmol/L (Fig. 3A), which was not toxic to RAW264.7 cells (Fig. 1). Treatment with 2.5 μmol/L fucoxanthin also induced apoptosis via the activation of caspase-3 (Fig. 3B, 4 and 5) in osteoclast-like cells. On the other hand, 2.5 μmol/L fucoxanthin did not decrease the cell viability of the osteoblast-like cell line MC3T3-E1 (Fig. 6), indicating that the apoptosis-inducing activity of fucoxanthin in osteoclasts is greater than that in osteoblasts. The present in vitro study suggests that fucoxanthin at certain concentrations suppresses osteoclastogenesis via the inhibition of osteoclast differentiation and the induction of apoptosis in osteoclasts, but that it does not deleteriously affect bone formation.

β-Cryptoxanthin, a carotenoid present in citrus fruits, has a potent inhibitory effect on osteoclast-like cell formation in mouse marrow cultures stimulated with RANKL, and no inhibition was observed in the presence of an inhibitor of protein synthesis, cycloheximide or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole [23]. Osteoprotegerin is a regulated suppressor of osteoclast differentiation [3] and is excreted from osteoblasts/stromal cells [32]. Uchiyama et al. [23] speculated that the inhibitory effects of β-Cryptoxanthin might be due to the inhibition of protein

synthesis such as via osteoprotegerin. In the present study, fucoxanthin also inhibited the RANKL-stimulated differentiation of RAW264.7 cells to osteoclast-like cells (Fig. 3A), but it is unknown whether RAW264.7 cells have the potency to express and excrete suppressor proteins such as osteoprotegerin. We speculate that the inhibitory effect of fucoxanthin in the present study may be unrelated to osteoprotegerin synthesis. Further studies are needed to verify the effects of fucoxanthin on protein synthesis such as its effects on osteoprotegerin expression.

Fucoxanthin induced apoptosis in osteoclast-like cells derived from RAW264.7 cells (Fig. 3B, 4 and 5). Our previous studies demonstrated that fucoxanthin also induced apoptosis in colon carcinoma WiDr and HCT116 cells [15] as well as hepatic carcinoma HepG2 cells [33]. Furthermore, it caused apoptotic cell death in prostate cancer PC-3, DU145, and LNCaP cells [34] and leukemia HL-60 cells [35]. Thus, fucoxanthin is able to induce apoptosis in several cell lines; however, the effective concentration differed among the cell lines, and the mechanism of its action is unknown. In a study of WiDr cells [15], the apoptosis induced by fucoxanthin might have followed cell cycle arrest because fucoxanthin caused cell cycle arrest at the G0/G1 phase through the up-regulation of p21 at low concentrations and induced apoptosis at high concentrations. A study of HL-60 cells [35] concluded that apoptosis induction by fucoxanthin may be mediated via mitochondrial membrane permeabilization and caspase-3 activation, but not involved with changes in the protein levels of Bcl-2, Bcl-XL, and Bax. The present study also indicated that fucoxanthin-induced apoptosis was accompanied by the activation of caspase-3 and the cleavage of PARP, a substrate of caspase-3 (Fig. 5). These results are consistent with the results of a previous study using HL-60 cells [35]. Probably, the apoptotic mechanism in osteoclast-like cells is similar to that in other cell lines as reported previously. In addition to fucoxanthin, β -Cryptoxanthin has also been reported to induce apoptosis in osteoclastic cells formed in a mouse marrow culture system *in vitro* [36, 37].

The present study demonstrated the inhibitory effect of fucoxanthin on

osteoclastogenesis in an in vitro system. However, the effects of fucoxanthin should also be discussed in terms of its bioavailability, and dietary fucoxanthin is considered to be hydrolyzed to fucoxanthinol in the gastrointestinal tract by digestive enzymes such as lipase and cholesterol esterase and then absorbed into intestinal cells [31]. Recently, we reported the distributions and concentrations of fucoxanthin and its metabolites in mice orally administered fucoxanthin [27, 38]. According to these studies, some dietary fucoxanthin was absorbed into the body without being metabolized and was detectable in the liver, lung, kidney, heart, spleen, and adipose tissue of the mice, but the concentrations of fucoxanthinol were higher than the concentrations of fucoxanthin in these tissues. Therefore, the bioavailability of fucoxanthinol is higher than that of fucoxanthin in the body. Furthermore, a previous study using human leukemia HL-60 cells [39] indicated that the apoptosis-inducing activity of fucoxanthinol was stronger than that of fucoxanthin. The present study also showed that > 60% of the fucoxanthin incorporated into osteoclast-like cells was hydrolyzed to fucoxanthinol, suggesting the active compound to induce apoptosis may be fucoxanthinol. Taken together, most dietary fucoxanthin may be converted to fucoxanthinol, and fucoxanthinol may exert a stronger suppressive effect on osteoclastogenesis than fucoxanthin do at the same concentrations.

Yamaguchi et al. [40] investigated the effects of marine algae on bone calcification in the femoral-metaphyseal tissues of rats. In their study, the marine algae were lyophilized and powdered, and rats were orally administered a 5% water suspension of the powder (1.0 mL per 100 g of body weight). Although the administration of a water suspension of *U. pinnatifida* or *E. bicyclis* powder increased calcium content in the femoral-metaphyseal tissues of rats, the bone calcium concentration did not increase in in vitro experiments using water-solubilized extracts from *U. pinnatifida* or *E. bicyclis* powder, indicating that the active compounds in *U. pinnatifida* and *E. bicyclis* are fat-soluble compounds such as carotenoids. Fucoxanthin is a carotenoid produced specifically by edible brown sea algae, and 11.1, 7.7, and 18.7 mg of fucoxanthin are present per 100 g of fresh raw *U. pinnatifida*

Suringar, *E. bicyclis* Setch. and *L. japonica* Areschoug sea algae, respectively [41]. Kanazawa et al. showed that ethanol concentrations of < 25% extracted less fucoxanthin from raw *L. japonica* Areschoug, whereas absolute ethanol (99%) gave the highest recovery ratio of fucoxanthin [41]. This indicates that it is impossible to extract fucoxanthin from *U. pinnatifida* and *E. bicyclis* powder. We speculate that the water-solubilized produced extracts of *U. pinnatifida* or *E. bicyclis* powder used in the previous study [40] contained less fucoxanthin, resulting in the lack of increase in bone calcium observed in their in vitro experiments. Therefore, the fucoxanthin in *U. pinnatifida* and *E. bicyclis* powder might have the active compound that increased bone calcification in previous in vivo experiments [40].

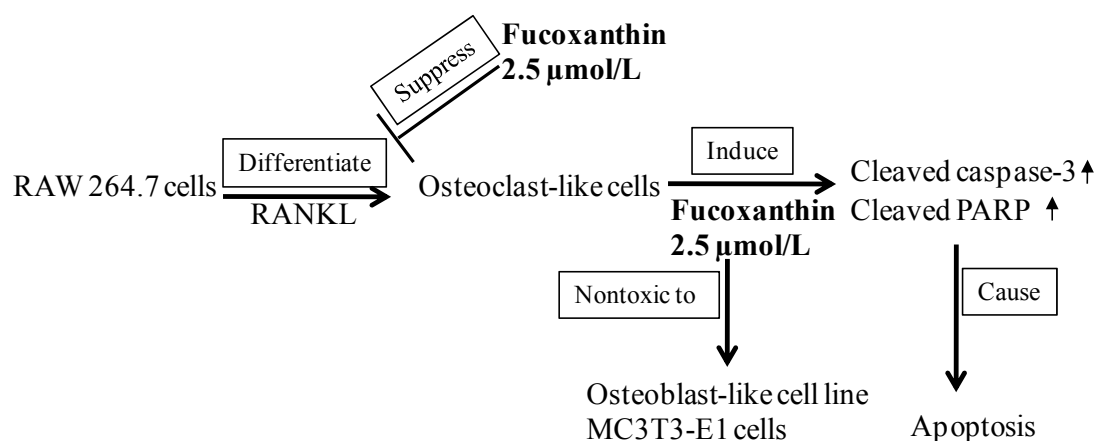


Fig. 8. Schematic representation of fucoxanthin on suppressing osteoclastogenesis and inducing osteoclast-like cells to apoptosis.

In conclusion, fucoxanthin at a concentration nontoxic to osteoblast-like cells suppresses the differentiation of RAW264.7 cells and it induces apoptosis in osteoclast-like cells by activation of caspase-3 and cleavage of PARP (Fig. 8). These results indicate that dietary fucoxanthin is helpful for the prevention of bone diseases such as osteoporosis and rheumatoid arthritis, which are known to be related to bone resorption.

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Chapter 3

Modulation of platelet aggregation-related eicosanoid production by dietary F-fucoidan from brown alga

***Laminaria japonica* in human subject**

3.1 Introduction

Brown alga *Laminaria japonica* is traditionally eaten in Japan as a beneficial food for health, and in chapter 2 the author found fucoxanthin from the alga suppressed osteoclastogenesis and induction of apoptosis in osteoclast-like cells, suggesting dietary fucoxanthin is helpful for the prevention of bone diseases. In addition to fucoxanthin, the other interesting compound in the alga is fucoidan. Fucoidan is a polysulfated polysaccharide found in various species of brown sea algae. The Japanese traditionally eat a large amount of sea algae and their life expectancy is the highest in the world [1]. Daily fucoidan intake from sea algae is estimated to be about 400 mg per capita [2, 3]. Fucoidan has been reported to have various biological effects, such as antitumor activity, by enhancing the immune response of hosts [4], anti-allergy activity by preventing NF- κ B p52-mediated pathways through CD40 [5], and anti-thrombosis activity in cell and animal experiments [6-8]; however, there is no information about the effect of dietary fucoidan and fucoxanthin on human thrombosis prevention and the mechanism is unclear. In this chapter, the author examines the both fucoidan and fucoxanthin on the thrombosis prevention in humans.

Thrombosis prevention is an important issue since thrombosis causes various abnormal conditions in the circulatory organs, such as vascular occlusion, transient ischemia, and infarction, which are closely associated with the induction of various degenerative diseases [9]. Thrombus formation is induced by platelet activation, and the ability of platelets to participate in both normal hemostasis and atherothrombosis depends on their adhesive properties and their capacity to be activated very quickly in response to various stimulus [10]. When activated, platelets secrete biologically active

ligands, including adenosine diphosphate, serotonin, and thromboxane A₂ (TxA₂), to induce thrombus formation [11] and platelets also release arachidonic acid from their plasma membranes to produce TxA₂ and prostacyclin (PGI₂) via the arachidonate cascade [12]. TxA₂ is a potent platelet agonist and vasoconstrictor that activates resting platelets, resulting in thrombus formation, while PGI₂ is a potent vasodilator and inhibitor of platelet aggregation [13]. Patients with atherosclerotic disease or pulmonary hypertension display high TxA₂ levels and reduced PGI₂ levels [14, 15]. On the other hand, H₂O₂ has been reported to cause vasodilatation by several mechanisms, including cyclooxygenase and cyclic adenosine monophosphate (AMP) in canine cerebral arteries [16], and phospholipase A₂ in porcine coronary microvessels [17]. Exogenous H₂O₂ also causes vasodilatation by opening several K channels, including K_{ATP} channels in cat cerebral [18] and rabbit mesenteric arteries [19]. Furthermore, H₂O₂ can diffuse in tissues and cross cell membranes [20], making it an interesting candidate for signaling involved in the immune response.

In the present study, a dietary dose of fucoidan, prepared from *Laminaria japonica*, was orally administered to healthy participants to assess its anti-thrombotic effects. Furthermore, the anti-thrombotic mechanism of fucoidan was investigated using a Transwell co-culture system, the Caco-2 intestinal epithelial model and human blood. This study provides new insight into the mechanism of fucoidan as an anticoagulant in humans.

3.2 Materials and methods

3.2.1 Preparation of fucoidan and fucoxanthin

The brown algae *L. japonica* was cultured in Hakodate city, Japan, and extracted with absolute ethanol. To use the ethanol extract and its residues for the human study, the fucoidan and lipid fractions were encapsulated after the following preparation. Briefly, the extract was dried, de-salted, and re-extracted with hexane. After removing the hexane, the material was composed of 46.5% fucoxanthin, 8.01% lipids, and

45.4% carbohydrates of mostly cellulose. It was then mixed with cyclodextrin to make fucoxanthin (FX) capsules. The residue of the ethanol extract was suspended in 30 volumes of 0.1 M acetic acid, which was then filtrated through No. 2 filter paper, neutralized with 0.1 M NaOH, precipitated with 70% ethanol, and centrifuged at 7500g. According to a determination using an antibody to F-fucoidan [15], the precipitate was composed of 63% F-fucoidan and 37% of various other polysaccharides, mainly including alginic acid. The salt and arsenic contents were 0.02% and 0.9 ppm, respectively. This precipitate was made for F-fucoidan (FD) capsules. The fucoxanthin+F-fucoidan (FX+FD) capsule was made from combining the two fractions. Each capsule contained 0.2 mg fucoxanthin and/or 80 mg fucoidan. In the following human study, 5 capsules (1.0 mg fucoxanthin and/or 400 mg fucoidan)/participant per day were employed.

3.2.2 Purification of F-fucoidan

For the cell culture experiments, F-fucoidan in the FD capsules was further purified as follows: The components of the FD capsule were dissolved in 20 mM sodium acetate buffer (pH 4.6) and applied to a TOYOPEARL-DEAE 650M column (2.0 × 20 cm; TOSOH, Tokyo, Japan). The column was washed with 0.5 M-NaCl to wash out alginic acid and fucoidan was eluted with 1.0 M-NaCl. The F-fucoidan was then precipitated with ethanol in a final concentration of 67% and washed twice with 70% ethanol. The purity of F-fucoidan was > 95% according to the determination of total sugar by the phenol-sulfate method [21]. Thin-layer chromatography (TLC) of F-fucoidan hydrolysates prepared by 1% trifluoroacetic acid degradation at 100 °C for 1 h indicated that the F-fucoidan was composed of fucose and sulfated fucose in a molar ratio of approximately 1:1. No appreciable amounts of galactose and uronic acid were detected in the TLC. Sulfate content of F-fucoidan was determined to be less than 45% by the rhodizonate method [22]. Apparent molecular size of the F-fucoidan was estimated to be 300 kDa by 1% agarose gel electrophoresis using DNA molecular marker (500bp DNA Ladder; Takara, Tokyo, Japan). These analyses indicated that the fucoidan extracted from *L. japonica* was classified as F-fucoidan of fucose-rich fucan.

3.2.3 Study design

This study involved 5 experiments, all of which were approved by the ethics committee of Kobe University Graduate School of Medicine (permission no. 615). Experiment 1 involved 5-week intake of FX+FD capsule, FX capsule or FD capsule, and the effects of F-fucoidan treatments on the thrombosis status of 24 healthy participants were evaluated. Experiment 2 tested the effect of stress on eicosanoid production using fresh blood of healthy volunteers who had not been administered any test samples. Experiment 3 examined eicosanoid production, hydrogen peroxide levels and F-fucoidan amount in the blood of 13 healthy participants after 5-week intake of FD capsules. In addition, the effect of low levels of H_2O_2 on eicosanoid production and platelet aggregation were investigated. Experiment 4 studied the fate of dietary F-fucoidan in the mouse digestive tract. Experiment 5 involved in vitro examinations of the blood of healthy volunteers who had not been administered any capsules, aiming to elucidate the mechanism by which F-fucoidan modulates eicosanoid secretion into the blood. To clarify this description, a flow diagram is presented in Fig. 1.

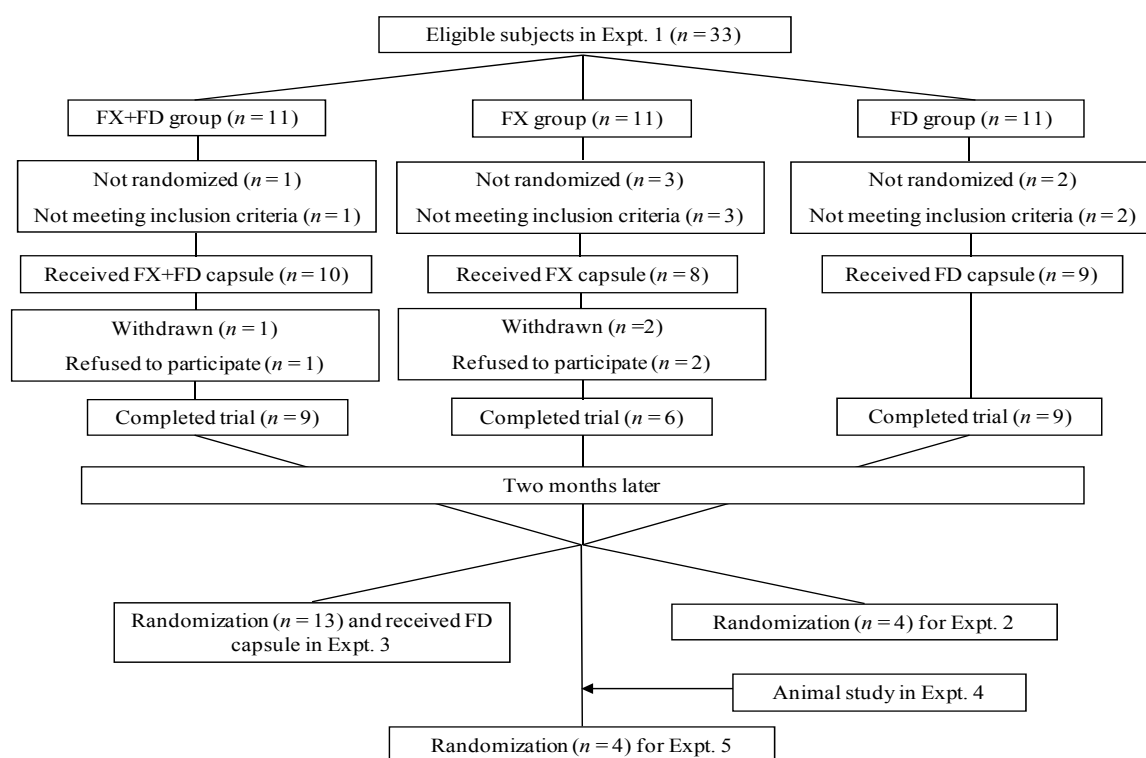


Fig. 1. Flow diagram of the study design

3.2.4 Participants

The participants were recruited from employees and students of Kobe University who were assessed as healthy in the regular health check at the health management center, and were excluded if they were taking medicine for hyperlipidemia, hypertension, diabetes, or hyperuricemia; if they had diet-controlled diabetes; if they were pre- or postmenopausal women or used oral contraceptives or hormone replacement therapy; if they were smokers or had a bleeding disorder, an allergy to algae, or a history of any gastrointestinal or cerebrovascular disease; or if they were using any dietary supplements. Informed consent was obtained from all eligible participants.

3.2.5 *In vivo* evaluation of the effects of fucoidan on thrombosis status (Exp.1)

First, 33 participants were randomly assigned to one of three groups containing 11 participants each, the FX+FD, FX and FD groups. The participants and investigators were blinded to which of the three groups each participant had been assigned to until the end of the study. At the baseline examination, blood was taken from participants and submitted to regular biochemical analysis (SRL Inc., Tokyo, Japan). Four participants were excluded because they were outside the normal range of 3.88-5.66 mmol/L total cholesterol (6.02, 6.41, 6.56, or 3.28 mmol/L), and two because they had caught cold and were taking medicine. After the 27 participants had started to receive 5 capsules per day for 5 weeks, three withdrew as they refused to participate because of the difficulty of taking 5 capsules daily. Finally, 13 male and 11 female participants were eligible and completed the study, nine in the FX+FD group (5 male, 4 female), six in the FX group (3 male, 3 female), and nine in the FD group (5 male, 4 female), as illustrated in Fig. 1. Their characteristics are shown in Table 1. Adherence to the intervention, which was evaluated by counting the remaining capsules in the bottle returned to the investigators and by examining a self-recorded dose diary, was > 98%. The participants continued their usual eating habits without any bias of eating algae during the 5-week experimental period.

At the baseline and final examinations, blood was carefully drawn from the

forearm by venipuncture through a 21-gauge butterfly cannula into a plastic syringe without using anticoagulants. The first 2 mL were discarded because the first blood had been subjected to stress by being churned in the syringe and would disturb the global thrombosis test (GTT) [23, 24]. The next 3 mL were used within 5 s for GTT, and another 10 mL were submitted for regular biochemical analysis.

3.2.6 GTT

The GTT was performed as described previously [25] to determine the occlusion time (OT) as a measure of platelet reactivity and lysis time (LT) as a measure of thrombolytic activity. Briefly, the above fresh blood was flowed spontaneously by gravity at 37 °C into a tube with a narrow end, which included two gaps formed by two steel balls, an initial large gap and a small point at the end. The blood undergoes shear force when passing through the gap made by the large ball, activates platelets to induce coagulation around the small ball, and then its flow is arrested. Drops of blood were detected optically with a light emitter and the recorded time when drops had not been detected for 15 s was referred to as OT to the start of blood coagulation. When blood flow was later restored with thrombolysis, the time of the first drop was referred to as LT to the start of lysis.

3.2.7 Estimation of the effects of shear stress on eicosanoid secretion (Exp.2)

To estimate the effects of stress on eicosanoid secretion, four participants were randomly selected and their fresh blood was taken 2 months after Exp. 1. After discarding the first 2 mL, 1 mL blood was subjected to shear stress by agitating it for 5 s with a vortex mixer or by incubating it for 40 min at 37 °C. After blood collection, serum samples were prepared by centrifugation and used for the determination of eicosanoid levels.

3.2.8 Measurement of eicosanoids

TxA₂ and PGI₂ have been well recognized to be immediately converted to thromboxane B₂ (TxB₂) after secretion and generally determined as equal products, thromboxane B₂ (TxB₂) and 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}), respectively [26, 27]. In the present study, the above 50 µL serum was analyzed with the general

method using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) [28].

The metabolite of TxA_2 was also determined in urine. Urine was collected from the participants in the morning before their breakfast at baseline and final examinations, and was submitted to analysis after removing impurities with centrifugation at 3000g at 4 °C for 5 min. The level of 11-dehydro-thromboxane B_2 (dTxB_2), a metabolite of TxA_2 , in urine was determined with an ELISA kit (Cayman Chemical) according to the manufacturer's instructions [6].

3.2.9 In vivo determination of eicosanoid production (Exp. 3)

Two months after Exp. 1, 13 participants, 7 male and 6 female, were randomly selected from the participants of Exp. 1. They were instructed to avoid brown sea algae-containing foods for the duration of the experiment and administered 400 mg FD capsules for 5 weeks, and then blood samples were taken at the baseline and final examinations. After discarding the first 2 mL, the next 2 mL blood samples were centrifuged at 3000g for 3 min at 4 °C, and the eicosanoid levels and fucoidan amount were measured in the serum. Also, H_2O_2 was determined with the following method.

3.2.10 Determination of H_2O_2

The serum level of H_2O_2 was determined using the scopoletin-fluorescence method [29]. A 100 μL aliquot of the serum was added to 100 μL Krebs Ringer phosphate buffer at pH 7.4 containing 5.5 mmol/L glucose, 17 $\mu\text{mol/L}$ scopoletin, and 0.44 units horseradish peroxidase and incubated at 37 °C for 30 min, before being subjected to analysis with fluorescence at 460 nm and excitation at 350 nm. The concentration of H_2O_2 was calculated using a standard calibration curve.

3.2.11 Effect of low H_2O_2 on human platelet aggregation

Platelet aggregation was determined by the published method [30]. Briefly, human blood in 3.2% of tri-sodium citrate was centrifuged at 200g for 15 min and separated into platelet-rich plasma (PRP). After confirming normal platelet numbers to be $2\text{--}4 \times 10^8$, the PRP was mixed with 2.5 $\mu\text{mol/L}$ H_2O_2 at 37 °C for 30 min and then placed in the wells of 96-well plates at a volume of 100 μL . After addition of platelet agonists (Sigma-Aldrich Co., Tokyo, Japan), 3.0 mg/L collagen, 0.5 $\mu\text{mol/L}$ arachidonic acid,

or 0.1 $\mu\text{mol/L}$ epinephrine, the 96-well plate was immediately placed in a microplate reader (SH-9000Lab; Corona Electric Co., Ibaragi Japan) shaken vigorously at 37 °C, and the absorbance monitored at 595 nm every 15 s for 15 min, because the absorption at 595 nm was maximum and proportional to the platelets aggregation [30]. In addition, a small part of the PRP was submitted to centrifugation at 15000g for 5 min and separated into platelet-poor plasma (PPP). The PPP was treated similarly to the PRP and was referred to as a control.

3.2.12 Study on fate of dietary F-fucoidan in mouse digestive tract (Exp.4)

The animal treatments were approved by the institutional Animal Care and Use Committee (permission numbers 20-5-11) and carried out according to the Guidelines on Animal Experiments of Kobe University. Male imprinting control region (ICR) mice (aged 7 weeks; Japan SLC, Shizuoka, Japan) weighing 28-30 g were acclimatized at 23 °C with a 12 h light-dark cycle for 1 week and were allowed *ad libitum* access to food (Rodent labo EQ 5L37; Japan SLC) and drinking water. The mice received a single oral administration of 5.0 mg/mouse of F-fucoidan after a 12 h fast and were then sacrificed at 2, 4, 8, 16, 32 h after administration. Blood and contents of the stomach, small intestine and large intestine, and feces were collected and submitted to an ELISA inhibition assay for measurement of F-fucoidan amount.

3.2.13 Co-culture system for evaluating eicosanoid secretion and H₂O₂ production (Exp. 5)

To elucidate the modulation mechanisms of dietary F-fucoidan on the production of eicosanoid in blood, an intestinal epithelial model was employed as described previously [31]. Human intestinal epithelial cell line Caco-2 cells were cultured in the upper well of a Transwell after being fully differentiated, which was measured by the method of Hidalgo *et al.* [32] with a Millicell-ERS (Millipore Co., Eschborn, Germany) exceeding 1200 Ωcm^2 trans-epithelial electrical resistance. Then, 0.8 mL fresh blood from healthy volunteers (n=4) was placed on the basolateral side and co-cultured with Caco-2 cells in the inserts. After 2 h co-culture, the medium in the inserts was replaced with medium containing 0, 125, 250, 500 mg/L fucoidan and

incubated for another 3 h. The blood was collected and the levels of H₂O₂ and eicosanoid were determined in the serum. Caco-2 cells in the insert were washed with PBS and subjected to real-time PCR to evaluate the effects of fucoidan on mRNA expression.

3.2.14 Real-time PCR

Total RNA in the Caco-2 cells was purified with a Mini RNA Isolation Kit using a capped column (Zymo Research Co.), as described previously [31]. Briefly, cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan). Real-time PCR was performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. TaqMan primers (assay ID: Hs0024659_m1, Hs00204187_m1, Hs02758991_g1; Applied Biosystems) were used to amplify human NADPH oxidase 1 (*NOX1*), dual oxidase 2 (*DUOX2*) and GAPDH, respectively. A real-time PCR 7500 fast system was used for the real time-PCR according to the manufacturer's instructions.

Table 1 Basal and final characteristics of participants (Exp. 1)^{1,2}.

Group	FX+FD				FX				FD			
	Basal		Final		Basal		Final		Basal		Final	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age, year	27.3	6.9			24.0	2.4			22.2	1.5		
Glucose, mmol/l	4.9	0.7	4.9	0.4	4.9	0.1	5.0	0.2	4.8	0.2	4.8	0.3
Total cholesterol, mmol/l	5.03	0.89	4.71	0.63	4.54	1.01	4.38	0.89	4.48	1.00	4.37	0.91
Aspartate transaminase, μ kat/l	0.32	0.06	0.33	0.08	0.35	0.01	0.34	0.02	0.35	0.13	0.34	0.07
Alanine transaminase, μ kat/l	0.25	0.10	0.26	0.10	0.25	0.07	0.25	0.04	0.26	0.17	0.26	0.17
Hematocrit, l	0.48	0.02	0.45	0.04	0.48	0.02	0.46	0.03	0.43	0.04	0.45	0.03
Red blood cell count, $10^6/l$	0.49	0.04	0.50	0.04	0.52	0.02	0.52	0.04	0.48	0.03	0.49	0.04
Platelets, $10^9/l$	208	46	234	43	223	16	229	22	223	35	229	25
Hemoglobin, g/l	152	15	152	13	155	10	153	13	149	14	151	13
Creatinine, μ mol/l	60	10	60	10	70	10	70	10	60	10	60	10
White blood cell count, $10^6/l$	5.2	0.8	5.0	1.2	5.3	1.1	5.5	0.5	5.8	1.1	5.7	1.6

¹ Values are presented as means $\pm$ SD, n=9 in FX+FD, n=6 in FX and n=9 in FD group, respectively.

² Blood was drawn after a 12-h fast.

3.2.15 Statistical analysis

The data are reported as the mean $\pm$ S.D. In Exp. 1 and Exp. 3, comparisons were made between baseline and final examinations in each group using the paired Student's t test. All other data were analyzed using the unpaired Student's t test. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Results of $p < 0.05$ were considered to be significant.

3.3 Results

3.3.1 Oral administration of F-fucoidan showed thrombolysis activity (Exp. 1)

No participants displayed any abnormalities or had any complaints at the end of the 5-week study period, and the background characteristics of the participants were not significantly different between the baseline and final examinations in any group (Table 1). OT did not significantly differ between the baseline and final examinations in any of the three groups, nor did LT in the FX group (Fig. 2). In contrast, LT in the FD and FX+FD groups was significantly shortened in the final examination compared with that at the baseline. As LT reflects the level of thrombolytic activity induced to de-aggregate platelets that have been occluded in blood vessels [23], the oral administration of F-fucoidan might be effective in preventing thrombosis.

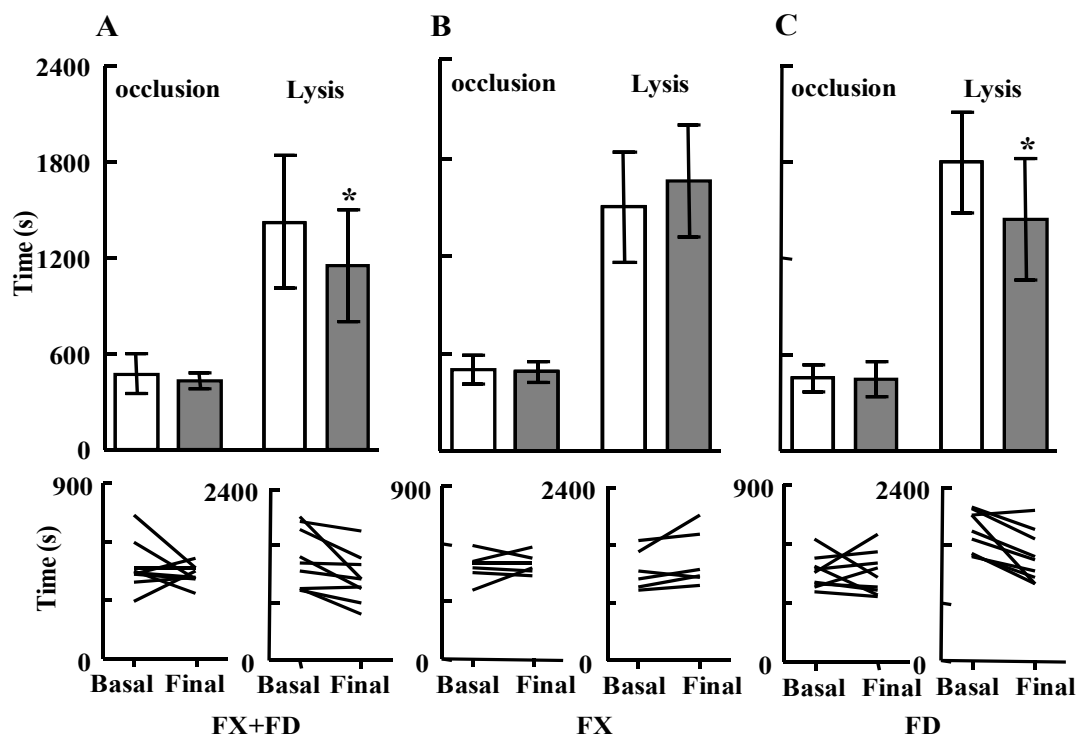


Fig. 2. Occlusion time and lysis time of blood at the baseline and final in a 5-week F-fucoidan ingestion study (Exp. 1). Fucoxanthin+Fucoidan (FX+FD) capsule group (n=9) (A), FX group (n=6) (B), and FD group (n=9) (C). Values in the upper panels are the means $\pm$ S.D., and those in the lower panels are individual items of raw data. * $P < 0.05$.

3.3.2 Inducing production of TxB_2 and 6-keto- $\text{PGF}_{1\alpha}$ by shear stress (Exp. 2)

The above GTT assay evaluates platelet aggregation induced by shear stress on the blood when it passes through the gap made by the large ball [23]. Shear stress has been reported to induce the secretion of TxA_2 , a potent platelet agonist and vasoconstrictor, and PGI_2 , a potent vasodilator and inhibitor of platelet aggregation [5, 33]. The half-lives of TxA_2 and PGI_2 have been reported to be very short, 30 s and 5 min, respectively, and metabolized to stable TxB_2 and 6-keto- $\text{PGF}_{1\alpha}$ [26, 27]. In Exp. 2, the change in serum levels of TxB_2 and 6-keto- $\text{PGF}_{1\alpha}$ was determined when fresh blood had undergone agitation for 5 s with a vortex mixer or had been left to stand at 37 °C, shown in Fig. 3. Stress by vortexing significantly increased the level of TxA_2 from 32 ± 11 ng/L to 94 ± 43 ng/L but 6-keto- $\text{PGF}_{1\alpha}$ remained unchanged (Fig. 3A). Leaving to stand at 37 °C markedly increased TxA_2 with time, from 61 ± 12 ng/L to 587 ± 24 ng/L after standing for 10 min, and 6-keto- $\text{PGF}_{1\alpha}$ increased slightly but

significantly with time (Fig. 3B). Thus, subjecting blood to stress increased the secretion of platelet aggregator TxA_2 from platelets or leucocytes.

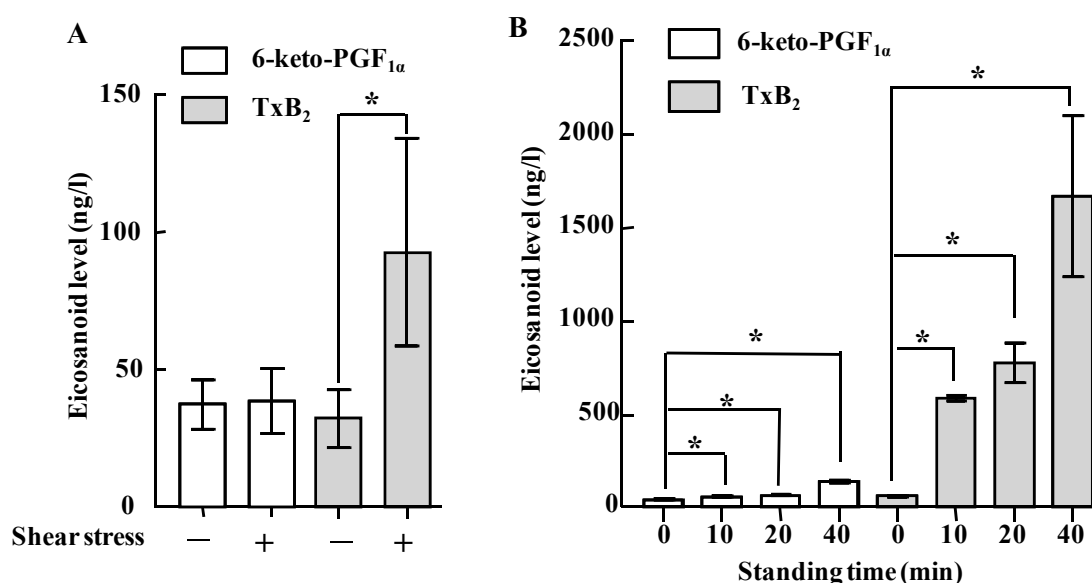


Fig. 3. Effects of stress on the production of TxB_2 and 6-keto-PGF_{1α} in serum (Exp. 2). (A) Fresh blood was treated with or without agitating and compared in the serum levels of TxB_2 and 6-keto-PGF_{1α}. (B) Fresh blood was left to stand at 37 °C to determine the serum levels of TxB_2 and 6-keto-PGF_{1α}. Value are the means $\pm$ S.D., $n=4$. Asterisks show a significant difference, $P < 0.05$.

3.3.3 Oral administration of *F-fucoidan* increased the serum level of 6-keto-PGF_{1α} and H_2O_2 (Exp. 3)

To examine the relevance of the *F-fucoidan* dose to eicosanoid production in blood, FD capsules were administered to 13 participants again for 5 weeks. At the end of administration, their blood was analyzed with an ELISA method and *F-fucoidan* was found to be under the detection limit ($< 2.5 \mu\text{g/L}$) in the serum (data not shown); however, the level of 6-keto-PGF_{1α} had significantly increased in the serum from $44 \pm 22 \text{ ng/L}$ at the baseline to $113 \pm 50 \text{ ng}$ (Fig. 4A). TxB_2 remained unchanged between the baseline and final examinations and had been reported to be excreted in urine after metabolism to dTxB_2 [6]. Morning urine was collected and analyzed at the baseline and final point. dTxB_2 was similar between the baseline and final point, $1426 \pm 624 \text{ ng/L}$ and $1452 \pm 847 \text{ ng/L}$, respectively. On the other hand, H_2O_2 is thought to induce vasodilatation through a mechanism including cyclooxygenase [8]. We therefore determined the serum level of H_2O_2 and detected a significant increase from

8.31 ± 1.43 to 9.76 ± 1.51 $\mu\text{mol/L}$. These results indicated that H_2O_2 may be involved in eicosanoid production.

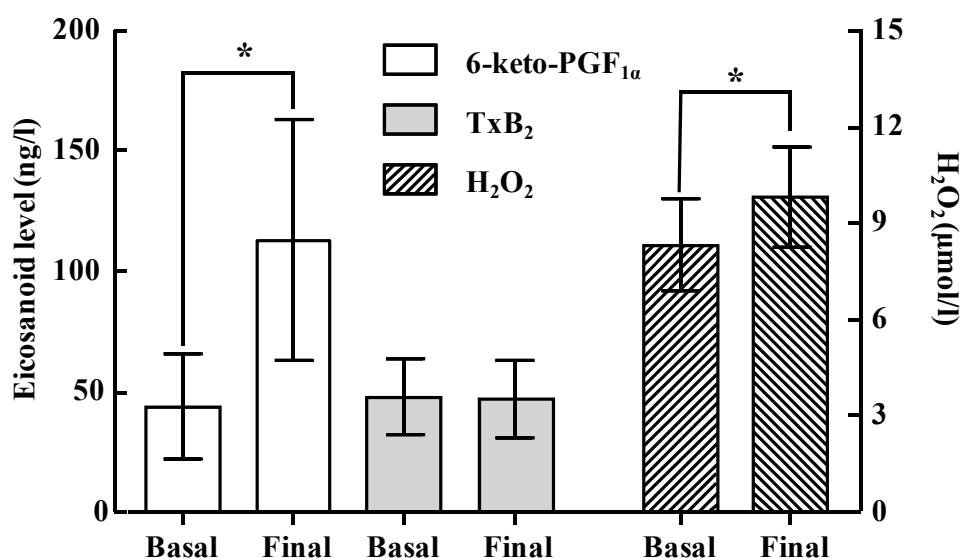


Fig. 4. Eicosanoid secretion and H_2O_2 production in serum after oral administration of F-fucoidan (Exp. 3). Thirteen healthy participants received 400 mg F-fucoidan per day for 5 weeks. The serum levels of 6-keto-PGF_{1α}, TxB₂, and H_2O_2 were compared in the basal and final experiments. Values are the means $\pm$ S.D., $n=13$. Asterisks show a significant difference, $P < 0.05$.

3.3.4 Effect of H_2O_2 on platelet aggregation

To examine the effects of H_2O_2 on the production of TxB₂ and 6-keto-PGF_{1α}, H_2O_2 was added to fresh blood, which was stressed by leaving to stand at 37 °C for 10 min or by agitating with 5 s vortexing, shown in Fig. 5. The addition of H_2O_2 increased 6-keto-PGF_{1α} in the stressed blood in a dose dependent manner, and TxB₂ remained unchanged (Fig. 5A). Production of both was inhibited by 100 $\mu\text{mol/L}$ aspirin, indicating that eicosanoid production is involved in cyclooxygenase [34] (Fig. 5A). H_2O_2 2.5 $\mu\text{mol/L}$ also increased 6-keto-PGF_{1α} in blood that had undergone shear stress (Fig. 5B). These results indicated that H_2O_2 modulated eicosanoid secretion in blood.

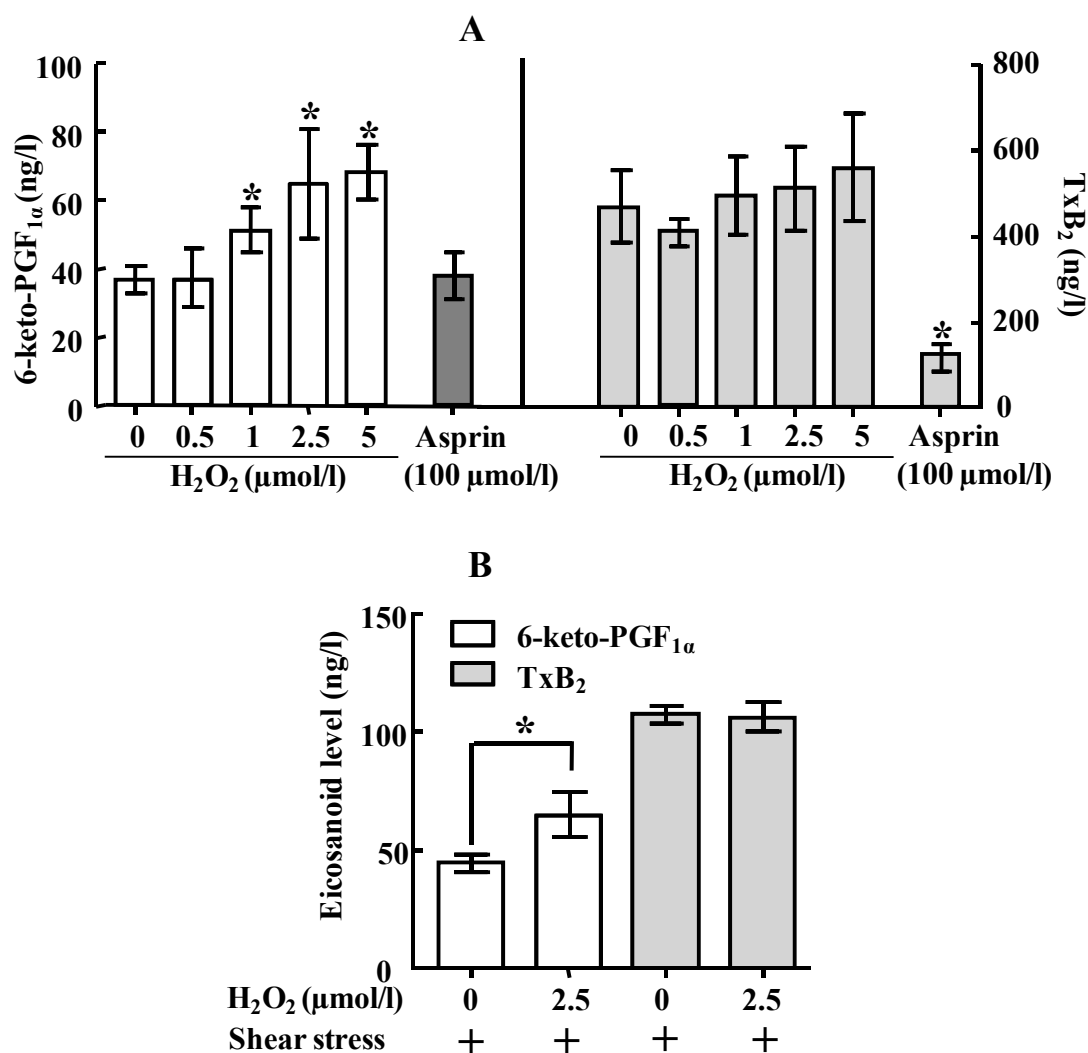


Fig. 5. Effect of H₂O₂ on production of 6-keto-PGF_{1α} and TxB₂ in blood. The indicated concentration of H₂O₂ or aspirin was added to fresh blood and subjected to stress by (A) leaving to stand at 37 °C for 10 min or (B) agitating by 5 s vortexing, and then TxB₂ and 6-keto-PGF_{1α} levels were determined in serum. Value are the means ± S.D., n=4. Asterisks show a significant difference from 0 μmol/L H₂O₂, $P < 0.05$.

Platelet aggregation has been reported to depend on the production of the platelet agonist TxA₂ in platelets [30]. In Fig. 5, H₂O₂ stimulated the production of platelet aggregation inhibitor PGI₂ at a concentration of more than 1.0 μmol/L. The effects of H₂O₂ were examined in Fig. 6. H₂O₂ suppressed platelet aggregation dose dependently (Fig. 6A) and significantly inhibited the aggregation induced by agonists [30] such as collagen, epinephrine and arachidonic acid (Fig. 6B). Thus, H₂O₂ plays an inhibitory role in platelet aggregation at a low concentration of around 2.5 μmol/L.

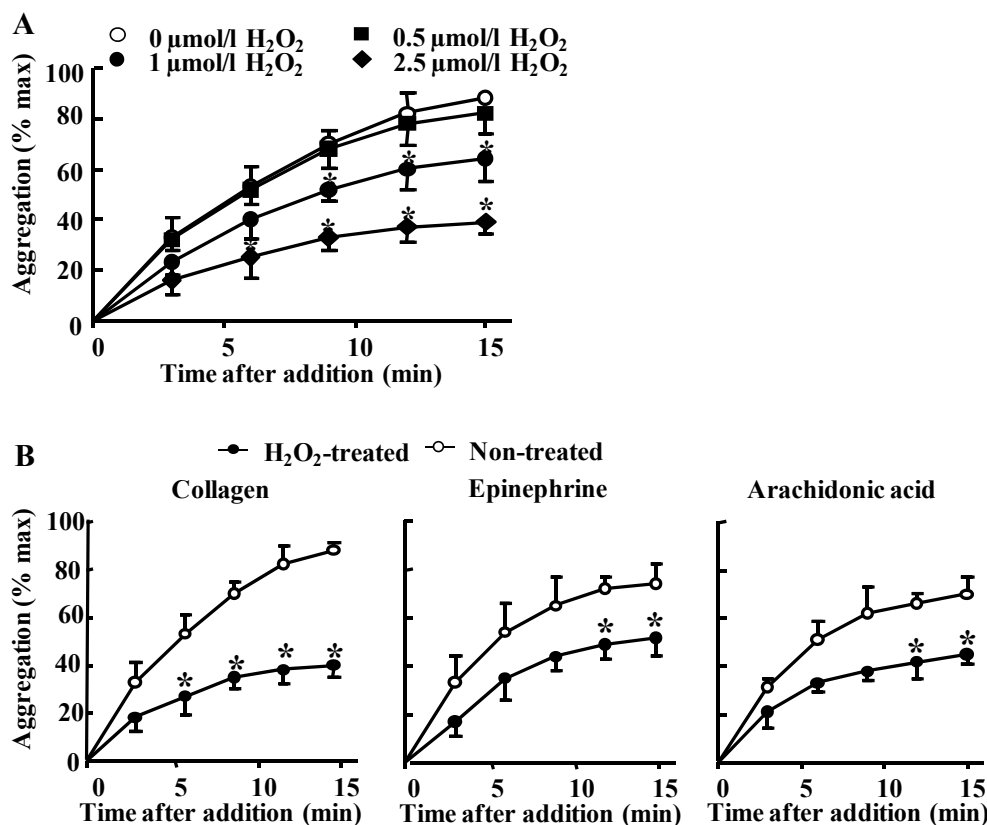


Fig. 6. Effect of H₂O₂ on platelet aggregation. (A) Platelet-rich plasma (PRP) prepared from the participant blood was treated with indicated concentration of H₂O₂ at 37 °C for 30 min and mixed with platelet agonist, 3.0 mg/L collagen; (B) PRP was treated with or without 2.5 $\mu\text{mol/L}$ H₂O₂ at 37 °C for 30 min and mixed with platelet agonists, 3.0 mg/L collagen, 0.5 $\mu\text{mol/L}$ arachidonic acid, or 0.1 $\mu\text{mol/L}$ epinephrine, and the absorbance was immediately monitored at 595 nm (with maximum absorption and proportional to the platelets aggregation) every 15 s for 15 min. For the blank control, platelet-poor plasma (PPP) was separated from some of the PRP by centrifugation and treated similarly to PRP. Value are the means $\pm$ S.D., $n=4$. Asterisks show a significant difference from each point 0 $\mu\text{mol/L}$ H₂O₂, $P < 0.05$.

3.3.5 Fate of dietary F-fucoidan in mouse digestive tract. (Exp. 4)

To know whether F-fucoidan could be incorporated into blood, 5.0 mg/mouse of pure F-fucoidan was dosed orally and its fate was examined in the digestive tract using a monoclonal antibody (Fig. 7). Dietary F-fucoidan in the stomach moved to the small and large intestines, and then all F-fucoidan was excreted in feces 32 h after dosing. In the blood, F-fucoidan was below the detection limit ($< 2.5 \mu\text{g/L}$) at every determination time. The results clearly showed that dietary F-fucoidan was not digested and was not incorporated into the blood.

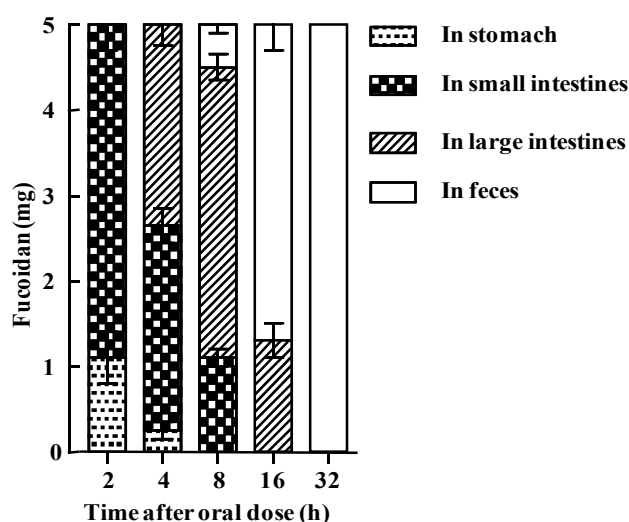


Fig. 7. Fate of dietary F-fucoidan in mouse digestive tract (Exp. 4). F-fucoidan 5.0 mg/mouse was orally dosed with a stomach sonde and traced in the digestive tract and blood with an ELISA inhibition assay at the indicated time after dosing. In blood, F-fucoidan was below the detection limit ($< 2.5 \mu\text{g/L}$). Value are shown as the mean $\pm$ S.D. (n=6).

3.3.6 F-fucoidan modulates eicosanoid secretion by stimulating Caco-2 cells to produce H_2O_2 (Exp. 5)

The above results indicated that dietary F-fucoidan increased H_2O_2 and modulated the secretion of TxA_2 and PGI_2 in blood serum although F-fucoidan was not incorporated into blood. To investigate the mechanisms of the effect of F-fucoidan, we employed an intestinal epithelial model of Caco-2 cells in the upper well and fresh blood in the lower well [35]. Before the investigation, the permeability of F-fucoidan through the Caco-2 monolayer was examined (data not shown). When F-fucoidan was added to the upper well for 3 h, it remained unchanged on the apical side and was below the detection limit ($< 2.5 \mu\text{g/L}$) on the basolateral side by detection with its antibody. Thus, F-fucoidan did not transfer to the blood side of the lower well.

The addition of 500 mg/L F-fucoidan to Caco-2 cells for 3 h significantly stimulated the expression of mRNAs of H_2O_2 -producing enzymes, *NOX1* [36] and *DUOX2* [37], in Caco-2 cells 1.62 ± 0.30 -fold (Fig. 8A) and 1.28 ± 0.05 -fold (Fig. 8B), respectively. Also, F-fucoidan increased the concentration of H_2O_2 on the blood side dose dependently (Fig. 8C), and 500 mg/L of F-fucoidan significantly elevated the level of 6-keto- $\text{PGF}_{1\alpha}$ in the serum while TxB_2 remained unchanged (Fig. 8D). These events were invalidated when F-fucoidan was added after mixing with its antibody (Fig. 8A-D), indicating that F-fucoidan itself directly affected Caco-2 cells. These results indicated that dietary F-fucoidan increased the production of H_2O_2 by stimulating the

expression of *NOX1* and *DUOX2* in intestinal epithelial cells and then modulated PGI₂ production in the blood.

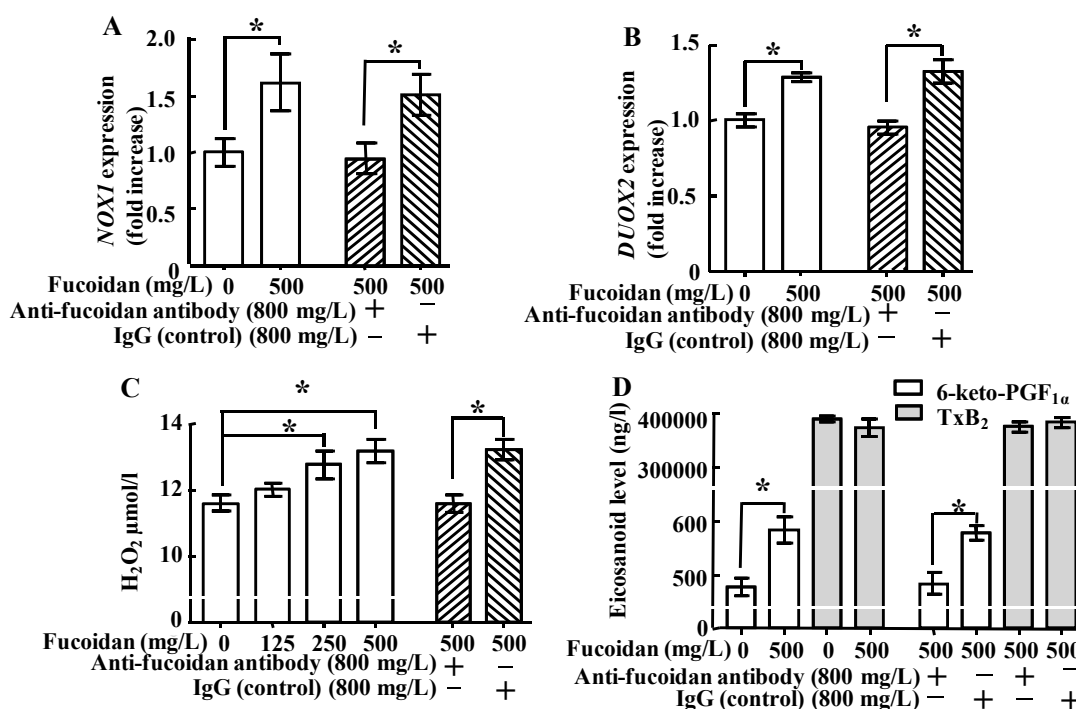


Fig. 8. Stimulating expression of *NOX1* and secretion of eicosanoid and H₂O₂ in Caco-2 cells after treatment with F-fucoidan (Exp. 5). Using the Transwell, Caco-2 cells in the upper well and fresh blood in the lower well were cultured for 2 h, and then the medium in the upper well was changed to medium containing the indicated concentration of F-fucoidan or F-fucoidan combined with its antibody. After another 3 h culture, the expression of (A) *NOX1* and (B) *DUOX2* in Caco-2 cells, and (C) the levels of H₂O₂ and (C) of TxB₂ and 6-keto-PGF_{1α} in the blood serum of the lower wells were determined. Value are the means $\pm$ S.D., $n=4$. Asterisks show a significant difference from 0 mg F-fucoidan, $P < 0.05$.

3.4 Discussion

The present study demonstrated that the intake of F-fucoidan from *L. japonica* shortened the LT of thrombus by elevating the secretion of PGI₂ as a result of increasing H₂O₂ production in blood. This is the first report to show that dietary F-fucoidan modulates platelet aggregation anti-thrombotically in humans.

Experiment 1 was a 5-week randomized and double blind study aimed at evaluating whether F-fucoidan has anti-thrombosis effects. Dietary fiber such as F-fucoidan is generally regarded to difficult to incorporate into body and their preventive effects on human have been suggested to involve their immune modulatory

activities in the intestinal epithelium [31, 38]. Assuming that a relatively long time will be required for the epithelium to respond to the action of dietary fiber, we decided that the period of oral administration should be 5 weeks. Three types of capsules, FX, FD and FX+FD, were prepared from *L. japonica* and dosed. FX is free of fucoidan and FD is free of fucoxanthin. Thus, FX is a placebo against FD, and FD is a placebo against FX.

The GTT assay can evaluate coagulating conditions of platelets when the blood undergoes shear stress that mimics the blood flow conditions induced by atherosclerotic plaque [24]. Under pathological conditions such as the rupture of atherosclerotic plaque or luminal stenosis, shear stress and flow disturbance account for most arterial thrombus formation [39]. Strony *et al.* [40] reported that shear stress is a risk factor for cardiovascular disease development because high stress occurs in 90% of stenosed coronary arteries. Thus, a shorter LT is closely associated with anti-thrombotic activity. In this study, the oral administration of fucoidan 400 mg/day for 5 weeks significantly reduced LT (Fig. 2), indicating that fucoidan may possess anti-thrombotic activity.

However, the signals that modulate the coagulation of platelets and their de-aggregation remain unclear. Thrombus formation involves three functionally independent pathways that mediate the interactions between circulating blood cells and the cells of the vessel wall: eicosanoids (such as PGI₂ and TxA₂), biological gases (such as NO and CO), ectonucleotidase (CD39) [4], and calcium ions [41]. Numerous reports have recognized that platelets play a pivotal role in arterial thrombus formation through TxA₂ and PGI₂ secretion [4-6, 26, 27]. Cheng *et al.* [42] have provided compelling evidence for the antithrombotic role of PGI₂ in vivo. The present study therefore focused on the eicosanoid pathway. Increasing production of eicosanoids was detected when the shear stress in the GTT test was reproduced in vitro using human blood (Fig. 3). F-fucoidan administration was performed again for 5 weeks in Exp. 3 and a significant increase was found in the serum level of 6-keto-PGF_{1α}, a metabolite of PGI₂ (Fig. 4). Thus, the intake of fucoidan was considered to

shorten LT by inducing PGI₂ production and would probably prevent cardiovascular diseases related to thrombosis.

The next question regarded which signals induced PGI₂ production. H₂O₂ is a reactive oxygen species and can react with various cellular targets, thereby producing toxicity that causes cell damage or even cell death. On the other hand, a low concentration of H₂O₂ is well known to play physiological roles, such as stimulating corneal epithelial cell adhesion to promote wound healing [43], having beneficial effects on post-ischemic myocardial recovery [44], and strongly enhancing the inhibitory effect of nitric oxide on platelet aggregation [45]. Sabetkar, M. *et al.* [46] reported that H₂O₂ at biologically relevant concentrations inhibited platelet function through increasing the serine phosphorylation of vasodilator-sensitive phosphoprotein. Shimokawa *et al.* [47] reported that H₂O₂ played an important role as a redox signaling molecule, causing vasodilation as well as cardioprotection. This indicates that H₂O₂ produces toxicity at a high concentration and plays a signaling role at a lower concentration, bordering on a specific threshold concentration. In the present Exp. 3, we detected an increase of H₂O₂ in blood after 5-week ingestion of F-fucoidan (Fig. 4). Then, we added a lower concentration of H₂O₂ to the fresh blood and found that H₂O₂ increased PGI₂ in both non- and stressed blood (Fig. 5). Furthermore, the addition of H₂O₂ reduced platelet aggregation induced by agonists (Fig. 6). These results indicate that H₂O₂ is a signal to modulate the secretion of the inhibitor of platelet aggregation, PGI₂, in blood.

An interesting question is how dietary F-fucoidan affects PGI₂ secretion in blood. In Exp. 3, F-fucoidan was not detected in participant blood after 5-week ingestion of F-fucoidan. In Exp. 4, the mouse study showed that dietary F-fucoidan was not incorporated into blood and was totally excreted in feces (Fig. 7). A relatively larger amount of F-fucoidan (175 mg/kg body weight) was dosed compared to the amount in the human study (6.7 mg/kg). Even with this large amount, the animals did not have diarrhea and did not incorporate F-fucoidan into the blood. Several kinds of fucoidan have been reported to undergo digestion and to be partly incorporated into blood. The

F-fucoidan used in the present study was composed of fucose-rich fucan and was clearly different type from G-fucoidan (galactose-containing fucan) and U-fucoidan (uronic acid-containing fucan) reported in other brown algae [41, 48-50]. We therefore employed an intestinal epithelial model to elucidate the mechanisms of the effects of unincorporated F-fucoidan. F-fucoidan stimulated the expression of *NOX1* and *DUOX2* in Caco-2 cells and simultaneously secreted H_2O_2 and increased PGI_2 in the blood (Fig. 8). *NOX1* and *DUOX2* are enzymes producing H_2O_2 , and *DUOX2* is expressed throughout the digestive tract [51]. H_2O_2 has been recognized to diffuse in tissues and cross cell membranes [12]. Thus, these findings clearly demonstrate that dietary F-fucoidan acts on intestinal epithelial cells and produces H_2O_2 to transmit signals to modulate eicosanoid production in platelets (Fig. 9).

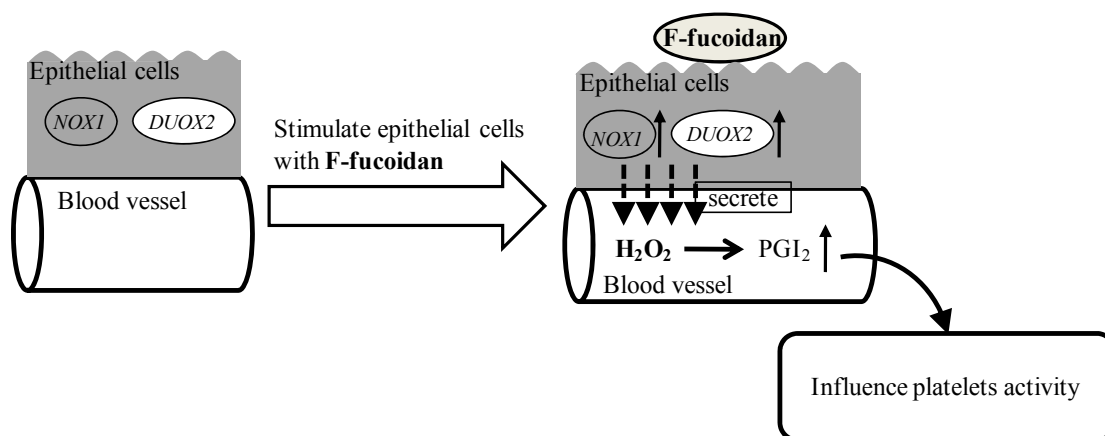


Fig. 9. Schematic representation of mechanism of F-fucoidan on thrombosis prevention.

Dietary *Laminaria* F-fucoidan markedly shortened LT in human blood. Daily intake of F-fucoidan might contribute to the prevention of cardiovascular diseases by suppressing thrombus formation.

3.5 Reference

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Chapter 4

9-Oxononanoic acid, lipid peroxidation product, is a primary signaling chemical to induce phospholipase A₂ activity and thromboxane A₂ production in human blood

4.1 Introduction

Although H₂O₂ is better known for its cytotoxic effects, in recent years it has become established as an important regulator of eukaryotic signal transduction. In chapter 3 the author found that fucoidan acted on intestinal epithelial cells to release low concentration H₂O₂, which played as a signaling molecule and suppressed thrombus formation. Most biological sources of H₂O₂ involve the spontaneous or catalytic breakdown of superoxide anions (O²⁻), produced by the partial reduction of oxygen during aerobic respiration. H₂O₂ and O²⁻ are oxygen-derived species and produced at a low level by normal aerobic metabolism. Normal cellular defense mechanisms destroy most of these; however, if a significant decrease in the effectiveness of antioxidant defenses, that will cause oxidative stress. It is an important cause of cell damage associated with the initiation and progression of many diseases. In this chapter, the author tried to confirm that oxidative stress increased 9-Oxononanoic acid (9-ONA) production which leaded blood to clot and initiated atherosclerosis.

Lipids with polyunsaturated fatty acids (PUFAs) are primary targets for attack by reactive oxygen species (ROS) [1], and the oxidation of the endogenous lipids is closely associated with various diseases, such as atherosclerosis, cancer, and the neurodegenerative disorders including Alzheimer's disease and Parkinson's disease. For example, in atherosclerosis [2], a number of oxidation products of lipids have been detected in atheroma [3] and oxidation of low-density lipoprotein (LDL) accompanies the disease process [4]. Many early studies have demonstrated that the

peroxidation products cause various degenerative diseases directly [5-7], while several studies have suggested that peroxidation products play a signaling role to induce the diseases [8, 9]. The former toxicity of peroxidation products has been almost clarified [10-12], but various questions remain about the latter signaling role of the products. In the present study, we were interested in the signaling role of peroxidation products.

In the signal transduction, phospholipids in cellular membranes play an important role in the control of cellular activities [13]. Hydrolysis of membranous phospholipids by phospholipase A₂ (PLA₂) releases free fatty acid including arachidonic acid from the *sn*-2 position of phospholipids, which is the start of arachidonate cascade and produces a precursor for essential cell-signaling eicosanoids. Arachidonic acid is converted by cytosolic prostaglandin G/H synthases and tissue-specific isomerases through unstable intermediate prostaglandin H₂ to multiple prostanoids such as thromboxane A₂ (TxA₂) and prostacyclin (PGI₂) [14]. TxA₂ is a potent platelet agonist and vasoconstrictor, and activates resting platelet, leading to the formation of a thrombus, while PGI₂ is a potent vasodilator and inhibitor of platelet aggregation [15]. Numerous studies have shown the important roles of TxA₂ and PGI₂ in preserving the dynamic balance among thrombosis, hemostasis and fluidity of blood. TxA₂ is high in patients with atherosclerotic disease and pulmonary hypertension, and the production of PGI₂ is depressed in these patients [16, 17]. Evidence that elevated PLA₂ activity is associated with cardiovascular events has also been found [18, 19]. Thus, the release of arachidonic acid by the PLA₂ from membranous phospholipids is the rate-limiting step in eicosanoid synthesis [20]; however, the signaling factors to activate the key enzyme PLA₂ are unknown. The aim of the present study was therefore to identify a candidate of the signaling chemicals to activate PLA₂.

Linoleic acid, a PUFA, has been reported to compose around 25% of the fatty acids of membranous phospholipids in human plasma [21]. The *sn*-2 position in membranous phospholipids is usually occupied by PUFA (22). Thus, linoleic acid is the most abundant PUFA in the *sn*-2 position of membranous phospholipids in human

plasma, and the major target of ROS is linoleic acid. ROS attack forms 9 or 13-hydroperoxide and is followed by production of various decomposed aldehydes. As shown in Fig. 1 [1, 22-24], 9-oxononanoic acid (9-ONA) is a major aldehyde. Previous studies showed that 9-ONA dosed orally significantly elevated the level of endogenous lipid peroxide [25], accompanied by marked reduction of lipogenesis in rat liver [8]. Furthermore, the stimulation of lipid peroxidation by 9-ONA was inhibited significantly by 71% with the addition of cyclooxygenase inhibitor, indomethacin or mepacrine, in rat hepatic microsomes [9]. These information indicated a possibility that 9-ONA is an inducer of arachidonate cascade through the activation of PLA₂. In the present study, we investigated the signaling role of 9-ONA in PLA₂ stimulation following by eicosanoid productions and platelet aggregation in human blood.

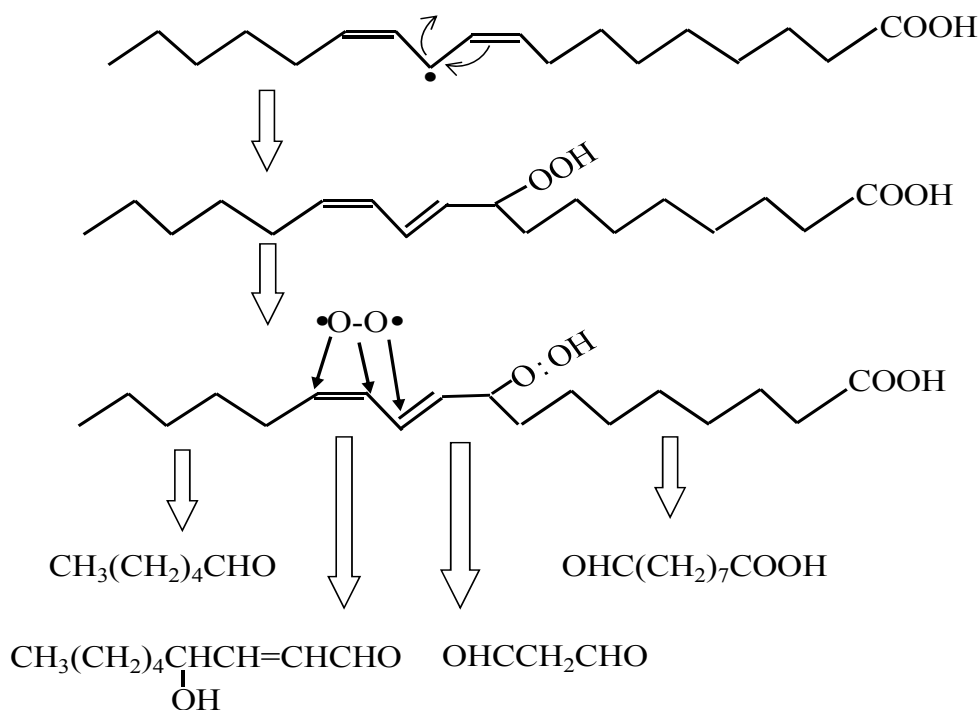


Fig. 1. Formation of linoleic acid 9-hydroperoxide and its hemolytic decomposition. Abstraction of hydrogen at 11 position accompanies the addition of oxygen to 9 or 13 position.⁽²³⁾ Resulting 9-hydroperoxide decomposes to various aldehydes after attack by another oxygen at one of the arrow positions; hexanal, 4-hydroxy nonenal, malonaldehyde, and 9-oxononanoic acid from left side in the figure.⁽²⁵⁾

4.2 Materials and Methods

4.2.1 *Materials.*

9-ONA was synthesized according to published methods [26]. Briefly, oleic acid was oxidized to dihydroxystearic acid using potassium permanganate and was oxidized further by potassium periodate to pelargonic aldehyde and 9-ONA. To separate 9-ONA from pelargonic aldehyde, the reaction products were submitted to silica gel column chromatography, and 9-ONA was eluted with a solvent of 60% diethyl ether in hexane after eluting pelargonic aldehyde by 20% diethyl ether in hexane. The 9-ONA was recrystallized from water 3 times and the pure was > 95% on HPLC, and was dissolved in ethanol just before using. The other reagents used were of the highest grade available from commercial sources.

4.2.2 *Human blood collection.*

This study was approved by the ethics committee of Kobe University Graduate School of Medicine (permission no. 615). Blood was carefully drawn from the forearm by fresh venipuncture through 21-gauge butterfly cannula into a plastic syringe without using any anticoagulants. The fresh blood was immediately applied to the following experiment.

4.2.3 *Autoxidation of human fresh blood.*

Fresh blood 5 mL each was placed in four open centrifugation tubes and incubated for 0, 10, 20, and 40 min at 37 °C, and then centrifuged at 3000g for 3 min at 4 °C. Four samples of 50 µL the serum layer were submitted to the following determinations; peroxide value (PV), thiobarbituric acid reactive substances (TBARS), PLA₂ activity, and TxB₂ level. The remaining both serum and plasma were submitted to extraction with 5 mL chloroform/methanol (2:1, v/v) three times according to the Folch method [27]. The chloroform layer was dried under nitrogen gas stream and the residue was dissolved in 500 µL methanol, and then 20 µL aliquot was analyzed by HPLC for determination of 9-ONA amounts.

4.2.4 Determination of lipid peroxidation in blood.

PV in above serum was determined according to published method [28]. Briefly, the 50 μ L serum was weighed into a 25 mL volumetric flask and mixed with 5 mL of chloroform and 10 mL acetic acid, and then de-aerated by N₂-gas purging for 1 min. The serum was mixed with 1 mL of 50% (w/v) KI aqueous solution and de-aeration for another 1 min, shaken and allowed to stand in dark for 5 min. The reaction mixture was diluted with 2% aqueous cadmium acetate solution and shaken, and then placed in the dark until the mixture had divided clearly into two layers. PV was calculated with the absorbance of aqueous layer at 410 nm using the following equation: $PV \text{ (meq/L)} = [(A-B) \times 60.14 + 0.69]/(0.008 \times V)$. Where A is the absorbance of sample, B is absorbance of blank, and V is the volume of sample (mL).

TBARS was determined by a fluorescent spectrophotometry according to the published method with slight modifications [29]. Briefly, the above 50 μ L serum was mixed with 20 μ L of 8.1% SDS, 0.15 mL of 20% acetic acid, and 0.15 mL 0.8% TBA, and then vortexed and incubated at 95 °C for 60 min. After cooling, the reaction mixture was combined with 0.5 mL butanol/pyridine (15:1) and centrifuged at 5000 rpm for 5 min. The upper butanol/pyridine phase was submitted to fluorescence analysis with an excitation wavelength at 520 nm and emission wavelength at 553 nm. TBARS was expressed as the levels of malonaldehyde after calculation with a standard curve constructed with tetraethoxypropane.

4.2.5 Effect of 9-ONA on PLA₂ activity and production of TxA₂.

Fresh blood 1 mL was placed in centrifugation tube which filled with nitrogen or not. After adding certain concentration of 9-ONA or not, it was incubated for the indicated time. The blood was centrifuged at 3000g for 3 min at 4 °C. From the upper layer 50 μ L was submitted to determine PLA₂ activity, and TxB₂ level.

4.2.6 Determination of PLA₂ activity in serum.

PLA₂ activity in the above 50 μ L serum was measured by a colorimetric method with commercial kit (Cayman Chemical, Michigan) according to the published method [30]. The reactions were initiated by an addition of substrate Arachidonoyl

thio-phosphorylcholine, and read changes in absorbance at 405 nm after 60-min incubation at 37 °C. Enzymatic activity was calculated with the manufacturer-provided formula and was expressed as $\mu\text{mol}/\text{min}/\text{mL}$.

4.2.7 Determination of TxA_2 level in serum.

TxA_2 has been well recognized to be immediately metabolized to thromboxane B_2 (TxB_2) after secretion and has been generally determined as equally produced TxB_2 [16]. The present study also adopted the general method, and the above 50 μL serum was measured with enzyme immunoassay kit (Michigan, Cayman Chemical) according to the published method [31, 32].

4.2.8 HPLC.

9-ONA was determined on HPLC system equipped with an L-7420 detector monitoring at a wavelength of 230 nm. The column (250 mm $\times$ 4.6 mm internal diameter) and guard column (10 $\times$ 4.0 mm internal diameter) were filled with Capcell pak C18 UG80 (Shiseido, Tokyo, Japan) and maintained at 35 °C. The mobile solvent was methanol/water (1:1, v/v), and the flow rate was 0.5 mL/min. 9-ONA was determined with calculation of the peak area on HPLC from calibration curve constructed with standard 9-ONA.

4.2.9 Effect of 9-ONA on platelet aggregation in human blood.

Human blood in 3.2% of tri-sodium citrate was centrifuged at 200g for 15 min and separated to platelet-rich plasma (PRP). After confirming normal platelet numbers to be $2\text{--}4 \times 10^8$ platelets, the PRP were placed in the wells of 96-well plates at the volume of 100 μL and mixed with various concentration of 9-ONA or platelet agonist 1 $\mu\text{mol}/\text{L}$, arachidonic acid, and then vigorously shaking at 37 °C on a microplate reader (SH-9000Lab, Corona Electric Co., Ibaragi Japan), changes in absorbance at 595 nm were monitored every 15 s for 15 min. Alternatively, a small part of the PRP was submitted to centrifugation at 15000g for 5 min and separated to platelet-poor plasma (PPP). The PPP was treated similarly to the PRP and was referred as to a control as shown in the published method [32].

4.2.10 Statistical analysis.

The data are reported as the mean $\pm$ standard deviation (SD). Statistical analysis was performed by SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) with the Dunnet post hoc test. Probability values less than 0.05 were considered as statistically significant.

4.3 Results

4.3.1 Production of 9-ONA in blood with autoxidation.

PV and TBA have been used generally for the evaluation of lipid peroxidation. PV specifically detects peroxides that are a primary product in lipid peroxidation [28], and TBA detects aldehydic products generated from decomposition of the lipid peroxides [33]. Fig. 2 showed that PV and TBARS increased with time when fresh human blood was exposed to atmospheric air. Thus, atmospheric oxygen induced lipid peroxidation in blood, and formed lipid peroxides and produced its decomposed products, aldehydes. After exposure to atmosphere for 40 min, blood gave various peak on HPLC (line c in Fig. 4), and one of the peaks coincided with authentic 9-ONA (line a in Fig. 4) with a retention times of 11.31 min. Blood combined with authentic 9-ONA showed that the peak at 11.31 min of retention time was enlarged and symmetrical with no shoulders (Fig. 4 line d), similar to the peaks in HPLC a, b and c. These results clearly indicate that the lipid peroxidation of blood produce 9-ONA in the blood. 9-ONA has been recognized to be one of the major aldehyde products in lipid peroxidation [24, 34], and Fig. 2A also showed that the level of 9-ONA increased in parallel with the increase of TBARS.

Fig. 3A showed the increase in PLA₂ activity with exposure time of blood to the atmosphere, and Fig. 3B showed the increase in TxB₂ and it was inhibited by 100 μ mol/L aspirin, which was a cyclooxygenase inhibitor [32] and had no suppressive effect on PLA₂ activity (Fig. 3A). Thus, PLA₂ activity and TxB₂ production increased following by lipid peroxidation in blood.

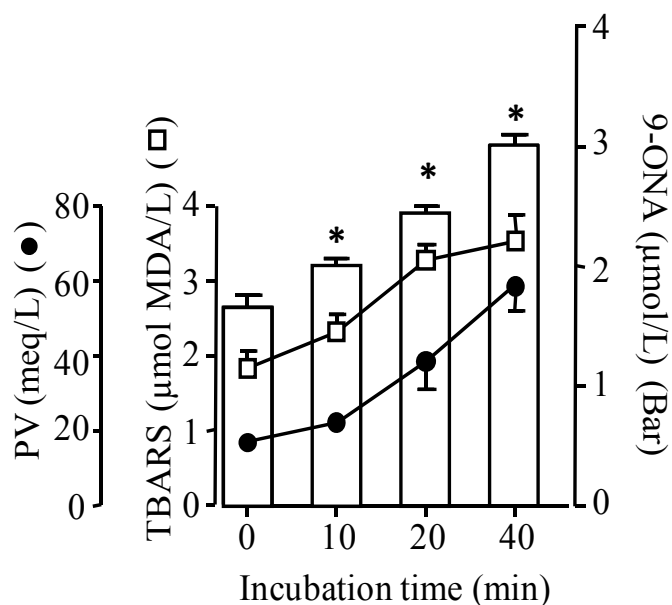


Fig. 2. 9-ONA production in autoxidation of blood and its accompanying changes in PV and TBARS. Human fresh blood was exposed to the atmosphere at 37 °C. After the indicated time, PV and TBARS in serum were determined, and also 9-ONA amount in whole blood was measured as shown in Materials and Methods. Value are mean $\pm$ SD, n=5. Asterisks are significant different from the values at 0 min, $P < 0.05$.

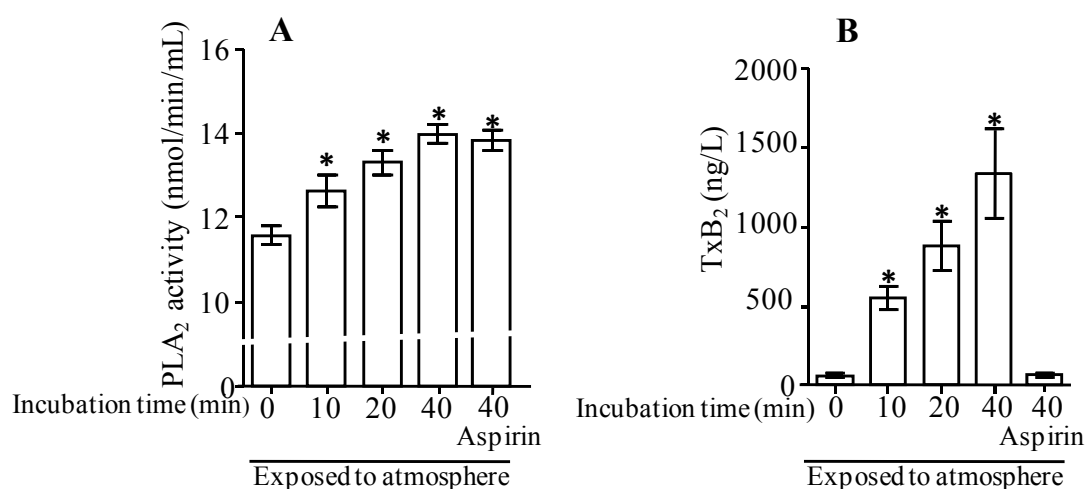


Fig. 3. Autoxidation of fresh blood increased PLA₂ activity and TxB₂ levels. Human fresh blood was added with or without 100 μmol/L aspirin and exposed to the atmosphere at 37 °C. After the indicated time, PLA₂ activity, and TxB₂ level in serum were determined as shown in Materials and Methods. Value are mean $\pm$ SD, n=5. Asterisks are significant different from the values at 0 min, $P < 0.05$.

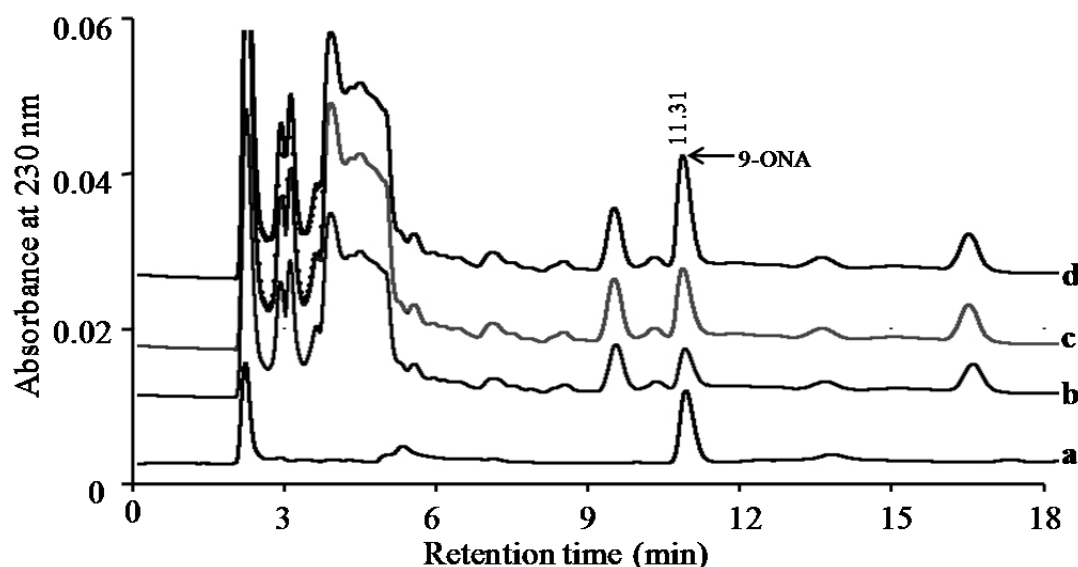


Fig. 4. Identification of 9-ONA in blood on HPLC. HPLC a: 17 $\mu\text{mol/L}$ of standard 9-ONA in methanol was applied to HPLC. HPLC b: fresh blood at 0 min in Fig. 2 was extracted with chloroform/methanol (2:1, v/v) as described in Materials and Methods; the concentration of 9-ONA was 11 $\mu\text{mol/L}$ in methanol. HPLC c: the blood exposed to atmosphere for 40 min in Fig. 2 was extracted and the concentration of 9-ONA was 19 $\mu\text{mol/L}$ in methanol. HPLC d: 17 $\mu\text{mol/L}$ of standard 9-ONA was added to the blood extract of HPLC c, and then analyzed.

4.3.2 Stimulating effect of 9-ONA on arachidonate cascade in blood.

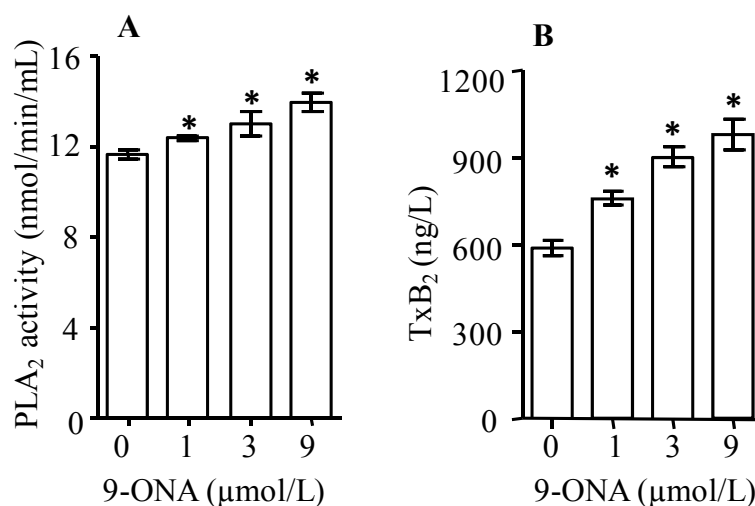


Fig. 5. Effect of organically synthesized 9-ONA on PLA₂ activity (A) and TxB₂ levels (B). Fresh human blood 1 mL was mixed with various concentration of 9-ONA and incubated at 37 °C in atmosphere for 10 min and was centrifuged at 3000g for 3 min at 4 °C. The serum part was submitted to the analyses of PLA₂ activity and TxB₂ levels with same methods to Fig. 2. Value are mean $\pm$ SD, n=5. Asterisks are significant different from the values with 0 $\mu\text{mol/L}$ of 9-ONA, P < 0.05.

The above results, the increases of PLA₂ activity and TxB₂ production followed

by lipid peroxidation, suggested that the peroxidation products was one of factors to stimulate the activity of PLA₂, the key enzyme of arachidonate cascade, and induce TxB₂ production. One of major products in the lipid peroxidation, 9-ONA, was added to fresh blood, and to PLA₂ activity and TxB₂ level determined in blood (Fig. 5). 9-ONA significantly increased PLA₂ activity (Fig. 5A) and TxB₂ level (Fig. 5B) in a dose dependent manner. The addition of 3 $\mu\text{mol/L}$ 9-ONA, a similar amount to that in blood when exposed to atmosphere for 10 min, increased PLA₂ activity by around 10% (from 11.6 ± 0.2 nmol/min/mL at control without 9-ONA to 12.9 ± 0.6 nmol/min/mL) and TxB₂ by around 50% (from 588 ± 27 ng/L of control 904 ± 36 ng/L). These results clearly showed that 9-ONA activated PLA₂ and induced production of TxB₂.

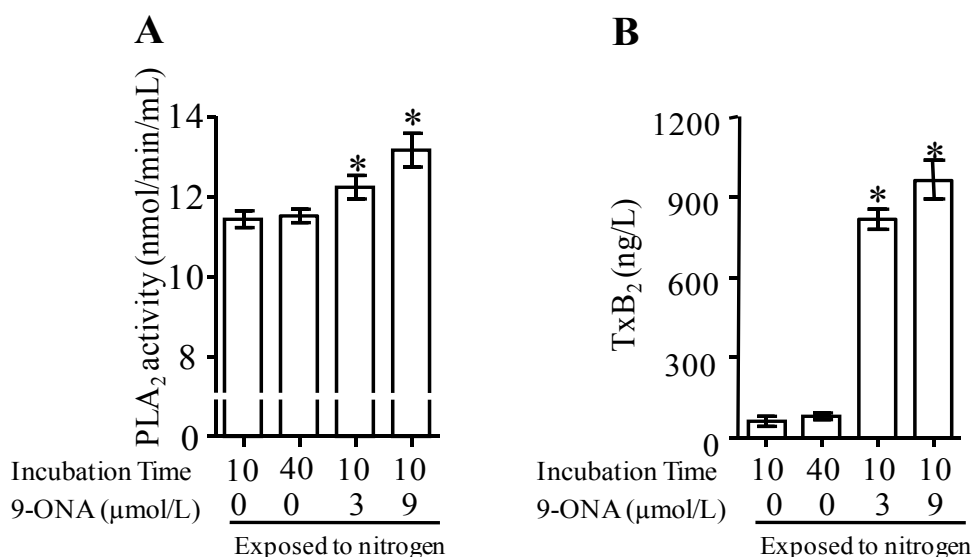


Fig. 6. Effect of 9-ONA on PLA₂ activity (A) and TxB₂ levels (B) on the condition of exposing to nitrogen. Fresh human blood 1 mL was placed in closed centrifugation tube which filled with nitrogen and was mixed with various concentration of 9-ONA. After incubating at 37 °C for the indicated time, the blood was centrifuged at 3000g for 3 min at 4 °C. The serum part was submitted to the analyses of PLA₂ activity and TxB₂ levels with same methods to Fig. 2. Value are mean $\pm$ SD, n=5. Asterisks are significant different from the values with 0 $\mu\text{mol/L}$ of 9-ONA for 10 min incubation, $P < 0.05$.

There was a question that whether inhibitor of the lipid peroxidation invalidated the activation of PLA₂ activity and TxB₂ production, thus inhibition experiment was done. Fresh blood was exposed to nitrogen gas instead of atmospheric air to prevent from lipid peroxidation, as shown in Fig. 6, there were no significant increase in both PLA₂

activity and TxB_2 production compared 10 min with 40 min. However, 9-ONA significantly increased the both in a dose dependent manner in the same condition exposing to nitrogen gas. These results confirmed that 9-ONA activated PLA_2 and induced production of TxB_2 .

TxA_2 is known to be an inducer of blood clotting, and production of TxA_2 in platelet was linear relationship with platelet aggregation in vitro [32]. TxB_2 is the stable metabolite of TxA_2 after plays a signaling role in platelet aggregation [16]. Fig. 7 showed that the addition of 9-ONA induced the platelet aggregation in blood time and dose dependently. The addition with 3 $\mu\text{mol/L}$ 9-ONA produced the similar aggregation activity in blood with 1 $\mu\text{mol/L}$ of arachidonic acid that is a potent platelet agonist [14]. The result indicates that 9-ONA induces platelet aggregation through producing TxA_2 in the blood.

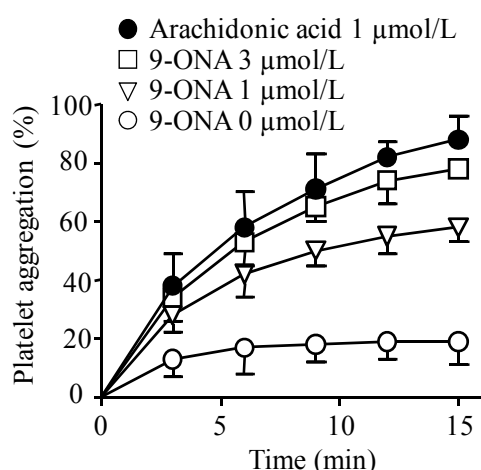


Fig. 7. Effect of 9-ONA on human platelet aggregation. PRP 100 μL in 96-well plate was treated with indicated concentrations of 9-ONA or platelet agonist 1 μmol of arachidonic acid. Aggregation was measured by changing absorbance at 595 nm (with maximum absorption and proportional to the platelets aggregation) every 15 s for 15 min with monitoring a plate reader shaking vigorously at 37 °C. All figures are $n=5$.

4.4 Discussion

The association of lipid peroxidation has been well recognized with numerous disorders such as inflammation, atherosclerosis and other degenerative diseases [2-5, 36]. Many previous works report that the peroxidation products of lipid, especially α , β -unsaturated carbonyls like 4-hydroxynonenal and malonaldehyde, attack the biological components and produce the toxicity [6, 7, 23, 37-39]. Thus peroxidation products directly cause the degenerative diseases; however, involvement in the induction of diseases has not been noticed in peroxidation products. The present study

demonstrates that one of the major products of lipid peroxidation plays a signaling role to initiate arachidonate cascade and stimulate production of eicosanoids, and then induce platelet aggregation.

The peroxidation of blood formed lipid peroxides of the primary product and produced its decomposed secondary products including various aldehydes, and one of the major secondary aldehyde products is 9-ONA (Fig. 2A). Additionally, aldehyde production is accompanied by the stimulation of PLA₂ activity and production of TxB₂ (Fig. 2B and C). PLA₂ is the key enzyme in the arachidonate cascade, and TxB₂ is the stable metabolite of TxA₂, one of the products of the arachidonate cascade [16]. This indicates that the lipid peroxidation in blood involves in the production of TxA₂ that plays a signaling role on platelet aggregation.

In the blood *in vivo*, lipid peroxidation is generally known to be initiated by ROS such as superoxide anion, which is generated in several systems such as neutrophil phagocytosis, oxidation of xenobiotics by cytochrome P450, xanthine oxidase and so on [40-43], and under clinical conditions such as shock, ischemia, and organ preservation [44, 45]. The primary targets of ROS attack are PUFAs [1], which usually occupy the *sn*-2 position in membranous phospholipid [46], and in human plasma, linoleic acid is most abundant at the *sn*-2 position [21]. In the peroxidation of linoleic acid, 9-ONA is one of the major aldehydic products, as shown in Fig. 1 [1, 22-24]. This indicates that 9-ONA is produced in human plasma when ROS occurs in the blood.

Among aldehydic products from lipid peroxidation, α , β -unsaturated carbonyls such as 4-hydroxynonenal, malonaldehyde and decadienal are highly unstable and easily form protein adducts [23, 37-39]. The peroxidation product must be stable when the product plays a signaling role in the biological system. 9-ONA is relatively stable in the blood since the accumulation of 9-ONA was detected in the peroxidized blood (Fig. 4). Then, 9-ONA was added to the fresh blood and examined for its signaling role and was found to stimulate the PLA₂ activity and TxB₂ production dose dependently (Fig. 5 and Fig. 6). Additionally, the addition of 9-ONA induced the

platelet aggregation dose and time dependently (Fig. 7). These results clearly showed that 9-ONA initiated the arachidonate cascade by stimulating key enzyme PLA₂ activity and aggregated the platelet by producing the signaling chemical TxA₂. Thus, the lipid peroxidation product 9-ONA is a stimulator inducing platelet aggregation, which is closely associated with atherothrombosis [47].

9-ONA stimulated the arachidonate cascade, which should produce not only TxA₂ but also prostaglandin I₂ (PGI₂). PGI₂ is a potent vasodilator and inhibitor of platelet aggregation [15]; however, PGI₂ was produced and released from macrovascular endothelium while TxB₂ was from platelet [48]. In the present study, blood was analyzed but human endothelium was not used. In order to examine PGI₂ production by 9-ONA, a human trial would necessary in a future study.

On the basis of the present results, the following speculation may be made: lipid peroxidation arises mainly in *sn*-2 position of membranous phospholipid of blood cells when any ROS is generated in the blood circulating system [1, 21]. Peroxidation produces various aldehydic products. Among them, α , β -unsaturated carbonyls easily form adducts with biological components [6, 7, 23, 37-39], and then relatively stable aldehydes accumulate in the blood. One of major accumulated products is 9-ONA which initiates the arachidonate cascade by activation of its key enzyme, PLA₂ activity. The arachidonate cascade produces TxA₂, which induces platelet aggregation and causes thrombus formation, and then develops the diseases such as atherosclerosis (Fig. 8). TxA₂ is immediately changed to TxB₂ after the agonist role in platelet. Thus, the present study provided new insight that lipid peroxidation product 9-ONA could play a signaling role to stimulate the key enzyme, PLA₂ activity, in the arachidonate cascade.

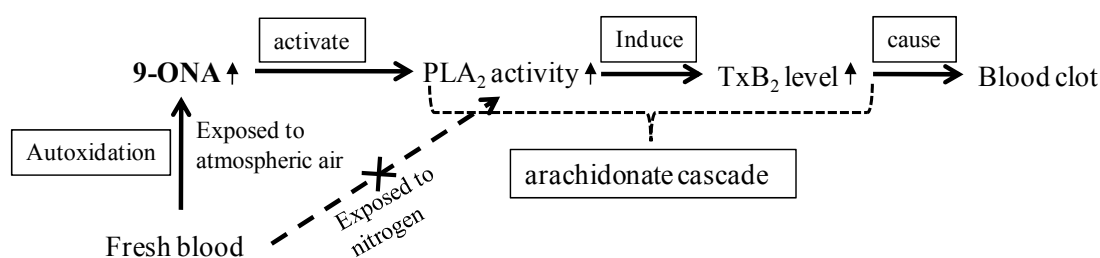


Fig. 8. Schematic representation of mechanism of 9-ONA on blood clot.

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Chapter 5

Conclusion

Life style related diseases usually emerge in middle age after long exposure to an unhealthy life style involving tobacco use, a lack of regular physical activity, and consumption of diets rich in highly saturated fats, sugars, and salt, and then it develops to chronic diseases. Diet has been known for many years to play a key role as a risk factor for chronic diseases. Diet and nutrition are important factors in the promotion and maintenance of good health throughout the entire life course. Their role as determinants of chronic disease is well established and they therefore occupy a prominent position in prevention activities (1). At the global level, great changes in diet have swept the entire world since the second half of the twentieth century. Traditional, largely plant-based diets have been swiftly replaced by high-fat, energy-dense diets with a substantial content of animal-based foods. The change in diet causes the increase of chronic diseases. It has been projected that, by 2020, chronic diseases will account for almost three-quarters of all deaths worldwide, and that 71% of deaths due to ischaemic heart disease, 75% of deaths due to stroke, and 70% of deaths due to diabetes will occur in developing countries (2). Indeed, cardiovascular diseases are even now more numerous in India and China than in all the economically developed countries in the world put together (3). Changing to a healthy diet containing function food such as kelp is a good approach to prevent life style related disease.

Japanese kelp, namely kombu is considered as longevity food and which have been consumed largely every year in Japan. Fucoidan and fucoxanthin are two unique compounds in the kelp and have been attracted much attention because of their disease preventing functions. In this study, the author explored the new chemopreventive function of the two compounds on anti-osteoporosis and anti-thrombosis. In chapter 2, the author showed that fucoxanthin did not produce any toxicity on RAW264.7 cells at the concentration up to 5 μ M, simultaneous addition of

fucoxanthin with RANKL to the culture systems significantly suppressed development of multinucleated osteoclasts in a dose dependent manner. Also, Fucoxanthin significantly inhibited the survival of osteoclasts after differentiated from RAW264.7 cells through inducing apoptosis by an activation of caspase-3, but did not affect on survival of pre-osteoblastic MC3T3 E1 cells. Thus, fucoxanthin seems likely to be preventive from bone-resorption related-diseases such as osteoporosis and rheumatoid arthritis.

Another treasure compound in kelp is fucoidan. Fucoidan has been reported to have various biological effects such as anti-tumor, anti-allergy activity. In chapter 3, the author investigated the anti-thrombosis activity in human and clarified the mechanism. The dose of F-fucoidan or F-fucoidan+fucoxanthin significantly shortened LT of the thrombus measured by a global thrombosis test in the blood, but fucoxanthin did not. To clarify the mechanism, the experiment on dose of F-fucoidan was repeated, the result showed dietary F-fucoidan increased H_2O_2 and the secretion of PGI_2 , a potent inhibitor of platelet aggregation, in the blood, but the amount of F-fucoidan was under the detection limit in the blood determined by ELISA assay. It indicates that F-fucoidan could not incorporate into blood. Furthermore, dietary F-fucoidan was totally excreted into feces when dosed to mouse and this result confirmed F-fucoidan was not incorporated into body. We then employed a co-culture system of a Caco-2 cell monolayer in the upper well with human fresh blood in the lower well to evaluate the mechanism of F-fucoidan. Addition of F-fucoidan to Caco-2 cells stimulated the expressions of *NOX1* and *DUOX2* mRNA and secreted H_2O_2 onto the blood side accompanied by a significant increase of serum PGI_2 production. These effects were invalidated by the combined addition of F-fucoidan with its monoclonal antibody. The results suggested that dietary F-fucoidan stimulated the expression of H_2O_2 -producing enzymes in intestinal epithelial cells and released H_2O_2 into blood, which played a signaling role to increase PGI_2 production and then shortened LT for thrombi.

H_2O_2 has become established as an important regulator of eukaryotic signal

transduction in recent years. Most biological sources of H_2O_2 involve the spontaneous or catalytic breakdown of superoxide anions (O^{2-}), produced by the partial reduction of oxygen during aerobic respiration. H_2O_2 and O^{2-} are oxygen-derived species and produced at a low level by normal aerobic metabolism. Normal cellular defense mechanisms destroy most of these; however, if a significant decrease in the effectiveness of antioxidant defenses, that will cause oxidative stress. It is an important cause of cell damage associated with the initiation and progression of many diseases. Thus, the author tried to confirm that oxidative stress increased 9-ONA production which led blood to clot and initiated atherosclerosis. In chapter 4, 9-ONA, one of major products from peroxidized linoleic acid was found to stimulate the activity of PLA_2 , which is the key enzyme for induction of arachidonate cascade and eicosanoid production. When fresh human blood was exposed to the atmosphere at 37°C , accumulation of 9-ONA was detected followed by an increasing peroxide value and thiobarbituric acid reactive substances in the blood. Lipid peroxidation was also accompanied by significant increases of PLA_2 activity and TxB_2 production, which is the stable metabolite of TxA_2 , a potent agonist for platelet aggregation. Organically synthesized 9-ONA was then added to fresh human blood. The 9-ONA significantly increased PLA_2 activity and TxB_2 production in a dose-dependent manner. Furthermore, 9-ONA induced the platelet aggregation dose dependently. These results indicate that a lipid peroxidation product, 9-ONA, is a primary signaling chemical to induce PLA_2 activity and the arachidonate cascade and to produce the agonist for platelet aggregation, TxA_2 , and is probably followed by the development of the diseases like thrombus formation. This is the first report to find that one of signaling chemicals, 9-ONA, stimulates the activity of a key enzyme in the arachidonate cascade, PLA_2 .

In conclusion, fucoxanthin in Japanese kelp contributes to the prevention of bone-resorption related disease by suppressing development of osteoclast or inducing apoptosis of osteoclasts. Dietary fucoidan in kelp contributes to the prevention of cardiovascular diseases by suppressing thrombus formation through arachidonate

cascade path way. These results may explain why kelp is considered as longevity food in Japan.

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List of Publications

1. Swadesh K. Das, **Rendong Ren**, Takashi Hashimoto and Kazuki Kanazawa. Fucoxanthin induces apoptosis in osteoclast-like cells differentiated from RAW264.7 cells. *J Agric Food Chem* **58**:6090-5; 2010
2. **Rendong Ren**, Yosuke Azuma, Takao Ojima, Takashi Hashimoto, Masashi Mizuno, Yosuke Nishitani, Masaru Yoshida, Takeshi Azuma and Kazuki Kanazawa. Modulation of platelet aggregation-related eicosanoid production by dietary F-fucoidan from brown alga *Laminaria japonica* in human subject. *Br J Nutr* (in press).
3. **Rendong Ren**, Takashi Hashimoto, Masashi Mizuno, Yosuke Nishitani, Hirosato Takikawa, Takeshi Azuma and Kazuki Kanazawa. 9-Oxononanoic acid, lipid peroxidation product, is a primary signaling chemical to induce phospholipase A2 activity and thromboxane A2 production in human blood. *J Clin Biochem Nutr* (in press).