Aim: We previously revealed that Ca$^{2+}$-activated calmodulin binds to ABCA1 by the region near the PEST sequence and retards its calpain-mediated degradation to increase HDL biogenesis. Calmodulin activity is reportedly modulated also by other nutritional divalent cations; thus, we attempted to determine whether Zn$^{2+}$ is involved in the regulation of ABCA1 stability through the modulation of calmodulin activity.

Methods: The effects of Zn$^{2+}$ on ABCA1 expression was investigated in J774 mouse macrophage cell-line cells and HepG2 human hepatoma cell-line cells.

Results: Zn$^{2+}$ increased ABCA1 expression, not by increasing the mRNA but by attenuating its decay rate, more prominently in the presence of cAMP. Accordingly, it enhanced cell cholesterol release with extracellular apolipoprotein A-I. Calmodulin binding to ABCA1 was increased by Zn$^{2+}$ and Ca$^{2+}$. Zn$^{2+}$ suppressed calpain-mediated hydrolysis of the peptide of ABCA1 cytosolic loop, including the PEST sequence and the calmodulin-binding site, in a calmodulin-dependent fashion, in the presence of the minimum amount of Ca$^{2+}$ to activate calpain, but not calmodulin. Calpain activity was not directly inhibited by Zn$^{2+}$ at the concentration for enhancing calmodulin binding to ABCA1.

Conclusion: Nutritional divalent cation Zn$^{2+}$ is involved in the regulation of ABCA1 activity and biogenesis of HDL through the modulation of calmodulin activity. The results were consistent with previous clinical findings that Zn$^{2+}$ increased plasma HDL in the conditions of sympathetic activation, such as type 2 diabetes and chronic hemodialysis.

Key words: Zinc, ATP binding cassette transporter A1, Calmodulin, Calpain, High density lipoprotein

Introduction

High-density lipoprotein (HDL) plays a central role in the catabolic pathway of cholesterol transport from the peripheral tissues to the liver for its conversion to bile acids. Moreover, it is considered an anti-atherogenic factor to prevent cholesterol accumulation in the vascular walls. HDL is biogenerated with helical apolipoproteins, such as apoA-I and cellular lipid, mediated by a membrane protein, ATP-binding cassette transporter A1 (ABCA1). In the organ-specific ABCA1 knockout study in mice, the liver was found to be the major source of plasma HDL, whereas the macrophage ABCA1 activity may be more directly responsible for atherogenesis, although its contribution to plasma HDL concentration is negligible.

ABCA1 gene expression is upregulated by the liver X receptor (LXR) sensing cellular cholesterol levels in general, and it is also negatively regulated by the factors such as protein kinase D and activator protein-2 system. This dual regulation may be more important in hepatocytes, where the ABCA1 gene expression is downregulated by sterol regulatory element-binding protein 2, in addition to the upregulation by LXR, perhaps to prevent backflow of cholesterol recovered from the extrahepatic tissues by HDL.

ABCA1 expression is also regulated by posttranslational proteolytic degradation by calpain following its endocytic internalization. This process is interfered by the interaction with helical apolipoproteins to enable ABCA1 resistance to calpain and hence...
enhance its recycling back to the surface\textsuperscript{15}. Caveolin-1 interacts with ABCA1 to facilitate its internalization and degradation, and probucol oxidative products interfere with this interaction to retard its internalization and degradation\textsuperscript{16-18}. Thus, ABCA1 expressed in cell surface seems responsible for this reaction and acts as one of the major rate-limiting factors for plasma HDL concentration\textsuperscript{19}.

Alternatively, calmodulin (CaM) interacts with ABCA1 at the region near the PEST sequence and retards its calpain-mediated degradation in a Ca\textsuperscript{2+}-dependent manner\textsuperscript{20}. CaM is a 16.8-kDa protein involved in calcium-signal transduction by interacting with various target proteins to modulate their functions that are activated by calcium ion\textsuperscript{21, 22}. Besides calcium, CaM has been shown to be activated by interacting with other divergent cations, such as La\textsuperscript{3+}, Tb\textsuperscript{3+}, Pb\textsuperscript{2+}, Sn\textsuperscript{2+}, Sr\textsuperscript{2+}, Hg\textsuperscript{2+}, Cd\textsuperscript{2+}, Zn\textsuperscript{2+}, and Mn\textsuperscript{2+} \textsuperscript{23-28}. CaM provides four calcium-binding sites, and these ions are thought to compete for these sites\textsuperscript{29}. Among these cations, Zn\textsuperscript{2+} has been suggested to act cooperatively with Ca\textsuperscript{2+} in some actions of CaM, such as activation of CaM-dependent protein kinase II\textsuperscript{30, 31}. Zinc is an essential micronutrient for animals; it is necessary for the maintenance of membrane structure/function and for the activities of over 200 metalloenzymes\textsuperscript{32}. Thus, it is crucial to investigate the effects of this metal nutrient on ABCA1 function for the regulation of plasma HDL metabolism. Many clinical studies indicated that zinc supplementation may decrease or induce no change in plasma HDL concentration. However, large-scale meta-analysis studies revealed that zinc supplementation increases HDL in normal subjects\textsuperscript{33-35}.

We thus examined the effects of Zn\textsuperscript{2+} on the stability and activity of ABCA1 through the modulation of the CaM function by using mouse macrophages and human hepatoma cell-line models in order to solve the controversial question on its effect on plasma HDL. Zn\textsuperscript{2+} increased ABCA1 by decreasing its degradation rate, more prominently in the presence of cAMP. It enhanced CaM binding to ABCA1 at the site near the PEST sequence to interfere with its calpain-mediated hydrolysis in the presence of the minimum amount of Ca\textsuperscript{2+} to activate calpain.

**Methods**

**Cell and Culture Conditions**

Mouse macrophage cell-line cells J774 were maintained in RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C\textsuperscript{36}. The cells were seeded in culture plates at a density of \(3 \times 10^6\) cells and cultured for 1 day before use. Human hepatoma cell-line cells HepG2 were cultured with DMEM medium (Sigma-Aldrich) containing 10% FBS. In a humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C\textsuperscript{39}, cells were seeded in culture plates at a density of \(2.5 \times 10^6\) and cultured for 1 day before use. Peritoneal macrophages were collected from 6-week-old male Balb/C mice, using 12 mice for one six-well plate\textsuperscript{37}. Ice-cold phosphate-buffered saline (PBS) (7 mL) containing 3% FBS was injected into the mouse peritoneal cavity followed by gentle massage of the peritoneum to dislodge any attached cells. The peritoneal fluid was collected and spun at 1500 RPM for 8 min to precipitate the cells. After discarding the supernatant and resuspending the cells in RPMI media containing 10% FBS, the cells were cultured at 37°C in 5% CO\textsubscript{2} for 2 h and washed with PBS once. The medium was changed to RPMI containing 10% FBS, and the cells were cultured at 37°C in 5% CO\textsubscript{2} for 48 h before use. HepG2 cells were pre-incubated for 18 h with DMEM containing 0.2% BSA and with and without 0.3 mM cAMP, washed with PBS, and incubated overnight in DMEM containing 0.1% BSA and various concentrations of ZnCl\textsubscript{2}, with and without 0.15 mM cAMP\textsuperscript{30}. The effects of divalent cations Ca\textsuperscript{2+} and Zn\textsuperscript{2+} were examined using various concentrations of CaCl\textsubscript{2} or ZnCl\textsubscript{2}. Normal extracellular concentration of Zn\textsuperscript{2+} in vivo may be 5 to 15 µM\textsuperscript{39, 40}, and its intracellular concentration is some 0.01 µM\textsuperscript{41}; the chosen range was 0.01–10 µM.

**Analyses of ABCA1 Protein**

Cell proteins were analyzed by immunoblotting. Cells were lysed in the lysis buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich) and subjected to SDS-PAGE and immunoblotting analysis by using the monoclonal antibody against ABCA1 (MABI98-7) generated in rats against peptide CNFAKDQS-DDDHLKDLSLHKN, a common sequence of the C-terminus of human and mouse proteins, at the MAB Institute (Yokohama, Japan), as previously described\textsuperscript{15, 16, 20, 42}. ABCA1 degradation was observed as a decrease in protein in the presence of cycloheximide to block protein synthesis, as previously described\textsuperscript{15, 16}. The experiments were conducted in the presence or absence of 0.15 mM cAMP.

**Real-Time Quantitative PCR**

The levels of ABCA1 mRNA was measured using synthetic oligonucleotide primers 5’-ACA ATACGT GTT GTA CGA ATG CCA GGG-3’, 5’-CTC ATC CTG TAG AAA AGA TGT GAG-3’ for J774 cells.
and 5'-GAA CTG GCT GTG TTC CA TGA T-3', 5'-GAT GAG CCA GAC TTC TGT TGC-3' for HepG2 cells, as previously reported17; in a 7300 Real-Time PCR System (Applied Biosystems). Total RNA was extracted using ISOGEN (Nippon Gene) and reverse-transcribed into cDNA using iScript™ cDNA Synthesis Kit (BIO-RAD). The cDNA was used as a template to produce PCR amplicats using SYBR PCR Permix Ex Taq Kit (TaKaRa).

Release of BODIPY-Cholesterol from Cells by Apolipoprotein (apo) A-1

ApoA-1 was isolated from human HDL fraction, as described previously43. The release of cell cholesterol by apoA-I was estimated by using fluorescence-lotting using an anti-ABCA1 antibody20). Ablintion of apoA-I was estimated by using fluorescence-lotting using an anti-ABCA1 antibody20). 

Apolipopr

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Apolipopr

CaM-Agarose Binding Assay

Lysate (200 µg as protein) of the cells pretreated with various concentrations of ZnCl₂ was incubated with 30 µl of 50% calmodulin-agarose (Merck) suspension in Tris-HCl saline for 3 h at 4°C. Protein bound to the beads was eluted with the SDS-PAGE sample buffer and analyzed for ABCA1 by immunoblotting using an anti-ABCA1 antibody20).

Calpain Reaction on the ABCA1 Peptide with the PEST and CaM-Binding Sequences

In order to examine the effect of CaM binding on calpain-mediated degradation of ABCA1, the peptide corresponding to 1213 to 1349 amino acid residues of ABCA1, including the PEST sequence and 1-5-8-14 motif of the CaM recognition sequence, was expressed as a fusion protein with glutathione S-transferase (GST) (GST–CaM–PEST) in Escherichia coli BL-21 DE3 (Nippon Gene). It was purified from the cell lysates and solubilized with 50-mmol/L Tris-HCl containing 10-mmol/L glutathione as previously described20. GST–CaM–PEST peptide, 2 µg, was incubated with 2 µg of CaM and 1.5 µg of calpain at 30°C for 45 min in the presence of various concentrations of Ca²⁺ and Zn²⁺, and the reaction products were analyzed by SDS electrophoresis.

Calpain Activity Assay in vitro

Calpain activity was measured using Calpain Activity Fluorometric Assay Kit (BioVision). Calpain and its substrate Ac-LLY-AFC provided by the kit were incubated under various concentrations of zinc in the presence of calcium. The fluorescence of the treated sample was examined in situ using a fluorescent plate reader (model SYNERGY/HTX Multi-Mode reader, BioTek) with excitation at 400 nm and emission at 505 nm.

Results

Effect of Zn²⁺ on ABCA1 Expression in Macrophages

Fig. 1 presents the effect of divalent cations on ABCA1 expression in J774 mouse macrophage cell-line cells. ABCA1 protein was increased by Ca²⁺, which is consistent with our previous finding20 (Fig. 1A). It was also increased by the incubation of the cells in the presence of Zn²⁺ (Fig. 1A). The increase was dose-dependent up to the concentration of 2 and 1 µM, respectively. In the presence of apoA-I that stabilizes ABCA1 protein against degradation, the effect of Zn²⁺ became somewhat fainter (Fig. 1A). Conversely, the mRNA level of ABCA1 was not influenced by Zn²⁺ (Fig. 1B). The degradation of ABCA1 was examined in the presence of cycloheximide (Fig. 1C). The half-life of ABCA1 was about 1 h in the non-treated cells, and it was apparently extended in the presence of apoA-I, which is consistent with our previous findings15, 16. The degradation rate of ABCA1 was also significantly retarded by Zn²⁺ with a half-life of 2–3 h in the absence of apoA-I. However, this effect was not apparent in the presence of apoA-I. ABCA1 expression was known to increase in the presence of cAMP45; thus, the effects of Zn²⁺ on ABCA1 expression was examined in the presence of 0.15 mM cAMP. The increase in ABCA1 and the retardation of its degradation were more significantly demonstrated in the condition cAMP is present (Fig. 2A, B). Similar findings were reproduced in mouse peritoneal macrophages in the absence and presence of cAMP (Fig. 2C). The activity of ABCA1 was evaluated by measur-
and presence of cAMP (Fig. 3B). The release of cholesterol from the cells was monitored both in the absence and presence of exogenous apoA-I, since hepatocytes produce HDL with endogenously synthesized apoA-I, presumably by an autocrine mechanism. Fig. 3C demonstrates the increase in cell cholesterol release by Zn²⁺ with and without exogenous apoA-I, both in the absence and presence of cAMP, which is consistent with the increase in ABCA1. Conversely, the message of ABCA1 was not influenced by Zn²⁺ (Fig. 3D).

**Interaction of CaM with ABCA1**

We previously reported that CaM interacts with ABCA1 in a Ca²⁺-dependent manner, and this interaction inhibits the calpain-mediated degradation of ABCA1, resulting in its stabilization and increase in its activity. We therefore examined the effect of Zn²⁺ on ABCA1 expression in hepatoma cell-line cells HepG2. ABCA1 was increased by Zn²⁺, both in the absence and presence of cAMP (Fig. 3A). The degradation of ABCA1 protein was substantially retarded by Zn²⁺, both in the absence and presence of cAMP (Fig. 3B). The release of cholesterol from the cells was monitored both in the absence and presence of exogenous apoA-I, since hepatocytes produce HDL with endogenously synthesized apoA-I, presumably by an autocrine mechanism. Fig. 3C demonstrates the increase in cell cholesterol release by Zn²⁺ with and without exogenous apoA-I, both in the absence and presence of cAMP, which is consistent with the increase in ABCA1. Conversely, the message of ABCA1 was not influenced by Zn²⁺ (Fig. 3D).
Effect of Zn\(^{2+}\) on calpain-mediated cleavage of ABCA1 peptide

We previously demonstrated that GST–CaM–PEST fusion protein (ABCA1 fragment peptide containing the CaM-binding motifs and the nearby PEST sequence) was completely degraded by calpain in the absence of CaM, and this reaction was inhibited in the presence of CaM and Ca\(^{2+}\). To analyze whether Zn\(^{2+}\) has the same mode of action, the reactivity of GST–CaM–PEST fusion protein to calpain was examined in vitro in the presence of CaM and Zn\(^{2+}\).
Finally, the direct effect of Zn\(^{2+}\) on calpain reaction is presented in Fig. 5, in which a commercial Calpain Activity Assay Kit was used in the absence of CaM. The reaction was inhibited by Zn\(^{2+}\) only at a concentration of 10 µM and higher, much higher than those for the effect observed in the presence of CaM. The results therefore demonstrated that Zn\(^{2+}\) suppresses calpain-mediated degradation of ABCA1 in a CaM-dependent manner at a concentration of around 1 µM. This is likely due to the enhancement of the binding of CaM to its binding site of ABCA1 to interfere with calpain-mediated degradation.

**Fig. 3.** The effects of Zn\(^{2+}\) on HepG2 cells

The cells were preincubated for 18 h, washed, and incubated with various concentrations of ZnCl\(_2\) with and without 0.15 mM cAMP. (A) ABCA1 protein expression was analyzed by Western blotting in the presence of increasing concentration of Zn\(^{2+}\) typically represented in the panel. The density of each band was quantified by using Photoshop software and standardized for β-actin for n=3. One-way ANOVA indicated a significant increase in ABCA1 by Zn\(^{2+}\) both in the absence and presence of cAMP (p<0.05). (B) Degradation rate of ABCA1. The effect of Zn\(^{2+}\) was evaluated by Western blotting. The cells were preincubated with 100 µg/mL cycloheximide for 60 min and then chased, and the level of ABCA1 was analyzed as a time course, as typically represented. Each band was analyzed for quantification by using Photoshop software. The values standardized for β-actin were shown relative to zero time of the time course and are expressed as mean±SD for three samples. The data showed significant difference with P<0.05 by the presence of Zn\(^{2+}\) at each incubation time point both in the absence and presence of cAMP. (C) The effect of Zn\(^{2+}\) on the release of cellular cholesterol. HepG2 cells were incubated for 1 h for labeling with BODIPY-cholesterol and equilibrated for 18 h in the absence and presence of 0.3 mM cAMP. The labeled cells were washed and incubated with various concentrations of Zn\(^{2+}\) in the presence of 10 µg/mL of apoA-I and 0.15 mM AMP for 4 h. The release of cellular cholesterol was estimated by measuring the fluorescence intensity of the medium. Data are expressed as mean SD for four samples, and the significance of the increase by Zn\(^{2+}\) is indicated as p-values by one-way ANOVA in each group. (D) The effect of Zn\(^{2+}\) on the expression of ABCA1 mRNA, by RT-PCR, as described in the text for n=3.
Zinc Ion Regulates ABCA1 by Modulating Calmodulin Activity

apoA-1-mediated cellular cholesterol was increased by Zn\textsuperscript{2+}, more prominently in the presence of cAMP; (3) the binding of CaM to ABCA1 was increased by Zn\textsuperscript{2+}, as shown by the co-precipitation of ABCA1 with CaM-agarose; (4) Zn\textsuperscript{2+} inhibited the calpain-mediated degradation of the ABCA1-peptide, including the CaM-binding site and the PEST sequence, in the presence of CaM. We concluded that Zn\textsuperscript{2+} cellular concentration is potentially involved in the regulation of ABCA1 activity by inhibiting its calpain-mediated degradation through the modulation of CaM activity.

Zinc is one of the essential inorganic metal nutrients involved in various cell functions. In vivo functions of zinc were originally described as its deficiency in animal studies by their symptoms, such as growth failure, hair loss, testicular atrophy, or thickening and hyperkeratinization of the epidermis\textsuperscript{46).} It later became recognized as an essential nutrient also for human beings, but its deficiency was thought to be rare earlier\textsuperscript{47).} It is now considered rather common, especially in developing countries\textsuperscript{48).} About two billion people worldwide are affected by zinc deficiency. Zinc deficiency is one of the leading causes of the loss of healthy life in developing countries, and it influences the elderly population even more in industrial countries, commonly appearing as a decrease in immune response or retardation of wound healing\textsuperscript{49).} Zinc deficiency may also be involved in the development of atherosclerosis through its influence on inflammation, gene stabilization and transcription, or apoptosis\textsuperscript{50-53).} However, the effect of zinc on lipid and lipoprotein metabolism is somewhat controversial in both animal models and humans. Zinc deficiency may push plasma lipoprotein profile towards atherogenic by increasing low-density lipoprotein\textsuperscript{35, 54), decreasing HDL\textsuperscript{35, 55), or

Fig. 4. Effects of Zn\textsuperscript{2+} on the ABCA1–CaM interaction
A: CaM-agarose beads were incubated with the 200 µg lysates of the cells preincubated at various concentrations of Ca\textsuperscript{2+} or Zn\textsuperscript{2+}. Protein bound to the beads was analyzed by Western blotting for ABCA1. B: Effect of Zn\textsuperscript{2+} on calpain-mediated cleavage of ABCA1 peptide. The peptide following ABCA1 sequence 1213 to 1349 that contains a CaM-binding site and PEST sequence tagged with GST (GST–CaM–PEST fusion protein) was examined for its proteolysis by \textmu-calpain under an increasing concentration of Ca\textsuperscript{2+}, analyzed by SDS-PAGE stained with Coomassie Brilliant Blue (CBB). C: Interference of calpain-mediated cleavage of ABCA1 peptide by CaM. GST–CaM–PEST protein was preincubated with (or without) CaM to allow it to bind to the 1-5-8-14 motif and then incubated with 0.5 µM Ca\textsuperscript{2+} (which allows activation of calpain but not of CaM) and 1.5 µg of \textmu-calpain for 1 h at 32 °C under various concentrations of Zn\textsuperscript{2+}. The reaction product was analyzed in SDS-PAGE stained with Coomassie Brilliant Blue (CBB).

Discussion

We previously reported that CaM interacts with ABCA1 in a Ca\textsuperscript{2+}-dependent manner, and this interaction inhibits calpain-mediated ABCA1 degradation to increase its activity for HDL biogenesis\textsuperscript{20).} On the basis of these findings, this study provided the following observations: (1) Zn\textsuperscript{2+} increased ABCA1 protein, but not the message, likely due to the retardation of its calpain-mediated degradation in both macrophage and hepatocyte cellular models; (2) the release of calpain activity by Zn\textsuperscript{2+} at high concentrations, such as 10 µM or higher, is possibly caused by competition against Ca\textsuperscript{2+} for calpain.

Fig. 5. Calpain activity assay
The direct effect of Zn\textsuperscript{2+} on calpain activity was examined. Active calpain (1 µg) was incubated with the calpain substrate (Ac-LLY-AFC) with various concentrations of Zn\textsuperscript{2+} (0.1 – 10 µM) at 37 °C for 1 h. Calpain inhibitor Z-LLY-FMK was used for a negative control incubation.
enhancing lipid peroxidation\textsuperscript{50}. Conversely, zinc supple-
tmentation was found to decrease HDL in normal
subjects while it increased in diabetic populations and
in those on chronic hemodialysis\textsuperscript{33-35}. No report has
been found on the molecular mechanism for these
findings.

ABCA1 is one of the major regulating factors of
plasma HDL concentration and is required in the biogene-
sis of HDL particles\textsuperscript{57} to regulate plasma HDL
concentration\textsuperscript{5} and atherogenesis\textsuperscript{6}. The activity of
ABCA1 is regulated by its gene transcription posi-
tively sensing cellular cholesterol and by its protein
degradation by various factors\textsuperscript{12, 15-17, 19, 36}. We found
that CaM is one of the factors involved in this pro-
cess\textsuperscript{20}. CaM is activated by Ca\textsuperscript{++} to interact with the
1-5-8-14 motif in the cytoplasmic loop of ABCA1
and thereby inhibits calpain-mediated degradation at
the nearby PEST sequence\textsuperscript{20}. CaM is known to be
driven not only by Ca\textsuperscript{++} but also by other divalent cat-
tions, such as La\textsuperscript{++}, Tb\textsuperscript{++}, Pb\textsuperscript{++}, Sn\textsuperscript{++}, Sr\textsuperscript{++}, Hg\textsuperscript{++}, Cd\textsuperscript{++},
Zn\textsuperscript{++}, and Mn\textsuperscript{++}\textsuperscript{22-28}. Among them, Zn\textsuperscript{++} seems to act
cooporarily with Ca\textsuperscript{++} rather than competitively in
some CaM actions, such as protein kinase II activa-
tion\textsuperscript{30, 31}. The results here with mouse macrophage
cell-line cells J774 indicated that Zn\textsuperscript{++} activates CaM
to interact with ABCA1 and retards its degradation to
increase HDL biogenesis. Schmitz and his colleagues
reported that zinc finger protein 202 acted as a tran-
scriptional repressor of ABCA1 and ABCAG1 and
suggested its involvement in the regulation of plasma
HDL\textsuperscript{58}. However, Zn\textsuperscript{++} did not exhibit any apparent
influence on the level of ABCA1 mRNA in the cur-
rent experimental conditions.

Clinical relevance of the current findings should
be carefully stated to avoid any overevaluation. While
many clinical studies suggested that zinc supplementa-
tion may not increase or even decrease plasma HDL
concentration, a large-scale meta-analysis demon-
strated that it decreases HDL in normal subjects but
increases HDL in type 2 diabetic populations and
chronic hemodialysis patients\textsuperscript{33-35}. No report has been
found on the molecular mechanism for these findings.
However, it should be noted that both of these condi-
tions are of sympathetic activation, where cAMP level
is to be increased in the target organ cells\textsuperscript{39-69}. The current
findings in fact indicated that cAMP enhanced the
effects of Zn\textsuperscript{++} on ABCA1 and the ABCA1-medi-
cated cellular cholesterol release in model cell culture
systems, both for macrophages and hepatocytes, which
are responsible for the atherogenesis and biogenesis of
plasma HDL, respectively. Zinc supplementation may
therefore be helpful to push lipid and lipoprotein
metabolism toward less atherogenic in certain patho-
logical states, although it is yet to be verified in appro-
priate animal models and by relevant clinical trials.

Acknowledgements

The authors are grateful to the undergraduate
students, Danki Ito, Naoto Kato, Riho Nakashima,
Mayu Yamamoto, Naoto Kobayashi, Kootmi Sakai,
Keishi Shimakura, Yurino Hattori, Kazuya Murata,
Yoichi Mitsumura, Goh Shonohara, Akane Ushikoshi,
Miho Nishio and Airi Yamamoto, who participated to
the preliminary stage of the project for their bachelor's
thesis. They also thank Dr. Shigeru Miyata at Chubu
University and Dr. Maki Tsujita at Nagoya City
University for his/her technical advices.

Funding Sources

This work has been supported by the MEXT-
Supported Program for Strategic Founding of
Research in Private Universities (S1201007) and by
Grants-in-aid from MEXT Japan (24614018,
26461370, 15H02903).

Clinical Interest and Disclosures

The authors have no affiliation with any organi-
zation with a direct or indirect financial interest in the
subject matter discussed in the manuscript.

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