International Journal of Cardiology 297 (2019) 111–117

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Anti-atherosclerotic effects of an improved apolipoprotein A-I mimetic peptide

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ARTICLE INFO

Article history:
Received 29 May 2019
Received in revised form 1 August 2019
Accepted 20 August 2019
Available online 22 August 2019

Keywords:
Atherosclerotic cardiovascular disease
Mimetic peptide
Cholesterol efflux

ABSTRACT

Background: Apolipoprotein (Apo)A-I is a major protein component of high-density lipoprotein (HDL) that causes cholesterol efflux from peripheral cells through ATP-binding cassette transporter A1 (ABCA1) and the generation of HDL. Furthermore, it has a possible protective function against atherosclerotic cardiovascular disease (ASCVD). We previously developed a novel ApoA-I mimetic peptide without phospholipids (Fukuoka University ApoA-I Mimetic Peptide, FAMP). According to our previous studies, FAMP had an anti-arteriosclerotic effect. Since the required dose and reaction time of conventional FAMP were relatively large and short, respectively, we newly developed an improved FAMP (i-FAMP).

Methods and results: We synthesized four candidate i-FAMPs, i-FAMP-D1, -D2, -D3 and -D4, and examined which i-FAMP has greater cholesterol efflux capacity than FAMP in A172 human glioblastoma cells transiently transfected with human ABCA1 cDNA. Only i-FAMP-D1 showed significantly greater cholesterol efflux capacity than conventional FAMP. i-FAMP-D1 formed stronger α-helical conformations than FAMP as assessed by circular dichroism spectra. Thus, we selected i-FAMP-D1 for further experiments. i-FAMP-D1 had a greater atheroprotective effect than FAMP in ApoE knockout mice. In addition, i-FAMP-D1 activated cholesterol efflux from macrophage to HDL more strongly than FAMP and increased cholesterol excretion from liver to feces.

Conclusion: These results suggest that i-FAMP-D1 has a stronger anti-atherosclerotic effect than conventional FAMP.

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1. Introduction

According to several large-scale randomized clinical trials, statin treatment for the primary and secondary prevention of atherosclerotic cardiovascular disease (ASCVD) prevents the onset and progression of ASCVD in one-third of cases, but not in the remaining two-thirds [1]. The relative residual risk of ASCVD is still a major problem after statin treatment. There are three major problems regarding the residual risk of ASCVD: 1) insufficient reduction of low-density lipoprotein cholesterol (LDL-C) levels, 2) low levels of high-density lipoprotein cholesterol (HDL-C) with dysfunctional HDL and elevated triglyceride (TG), and 3) insufficient control of other risk factors (high blood pressure, obesity, metabolic syndrome, type 2 diabetes, etc.) [2,3].

HDL functionality is involved in cholesterol efflux, as well as anti-oxidation, anti-inflammation, anti-proliferation and anti-thrombosis [4,5]. HDL mainly enhances reverse cholesterol transport (RCT), in which HDL takes up peripheral cholesterol and transfers it to the liver for excretion in the bile and feces. Recent prospective studies revealed that cholesterol efflux capacity was inversely associated with the incidence of CV events, restenosis rates after coronary stent implantation and graft failure in renal transplant recipients [6–8].

Although cholesteryl ester transfer protein (CETP) inhibitors significantly increased HDL-C levels in blood, CETP inhibition provides insufficient cardiovascular benefit [9,10]. Thus, there are currently only a limited number of therapeutic options to increase and enhance the function of HDL, although HDL is a target in the treatment of ASCVD. As an exciting new strategy, HDL therapy using reconstituted (r)HDL and apolipoprotein (Apo)A-I mimetic peptides has recently been developed [11–18]. ApoA-I mimetic peptides have been reported to exhibit many

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https://doi.org/10.1016/j.ijcard.2019.08.043
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of the atheroprotective biological functions attributed to HDL and can modify atherosclerotic disease processes in animal models. Recently, we developed a Fukuoka Apo A-I Mimetic Peptide (FAMP, without phospholipids), which consists of 24 amino acids as a modified ApoA-1 consensus sequence and exerts an anti-atherosclerotic effect through the enhancement of RCT [15,16]. In addition, FAMP protected against myocardial ischemia-reperfusion injury and promoted recovery from hindlimb ischemia through a NO-related pathway [17,18]. However, the dose and reaction time of FAMP were relatively large and short, respectively.

To resolve these issues, we developed new four candidate improved FAMPs (i-FAMPs) and examined which had greater cholesterol efflux capacity than conventional FAMP. In addition, we evaluated whether the candidate i-FAMP activated cholesterol efflux from macrophage to HDL more strongly than conventional FAMP and increased cholesterol excretion from liver to feces.

2. Methods

2.1. Materials

Human ApoA-I, HDL, and endotoxin-free bovine serum albumin (BSA) were purchased from Calbiochem. Synthetic liver X receptor (LXR) agonist T090131 and retinoid X receptor (RXR) ligands 9-cis-retinoic acid and 8Br-cyclic adenosine monophosphate (cAMP) were purchased from Sigma. Fmoc-amino acids and coupling reagents were purchased from Watanabe Chemical Ind. Ltd. A goat polyclonal anti-human ApoA-I antibody was purchased from Sekisui Medical Co, Ltd.

2.2. Peptide synthesis

FAMP and i-FAMPs were synthesized by Fmoc (N-[9-fluorenyl] methoxycarbonyl)-based solid-phase peptide synthesis using an automated peptide synthesizer (Focus XC, AAPPTec) with the standard Fmoc methodology as described previously [15,19].

2.3. Cell preparation and cultures

A172 human glioblastoma cells and J774 macrophages (Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM containing 10% fetal bovine serum (Life Technologies Japan Ltd), 100 units/mL penicillin G, and 100 μg/mL streptomycin. For all experiments, cells were maintained in serum-free medium containing 0.2% BSA with or without additives (5 μmol/L T0901317 and 9-cis-retinoic acid or 0.3 mmol/L 8Br-cAMP).

2.4. Cellular cholesterol efflux

A172 human glioblastoma cells transiently transfected with human ABCA1 cDNA using Lipofectamine 2000 reagent (Invitrogen) and J744 macrophages were used for cholesterol efflux experiments. The cells were radiolabeled with ^{3}H-cholesterol, and cellular cholesterol efflux was measured as described previously [20].

2.5. Characteristics of i-FAMP-D1 as assessed by matrix-assisted laser desorption/ionization - Time-of-Flight Mass Spectrometry (MALDI-TOF-mass), high performance liquid chromatography (HPLC) and circular dichroism (CD) spectra

The characteristics of i-FAMP-D1 after purification were determined by MALDI-TOF-Mass and HPLC. CD spectra of i-FAMP-D1 and FAMP in water were also analyzed.

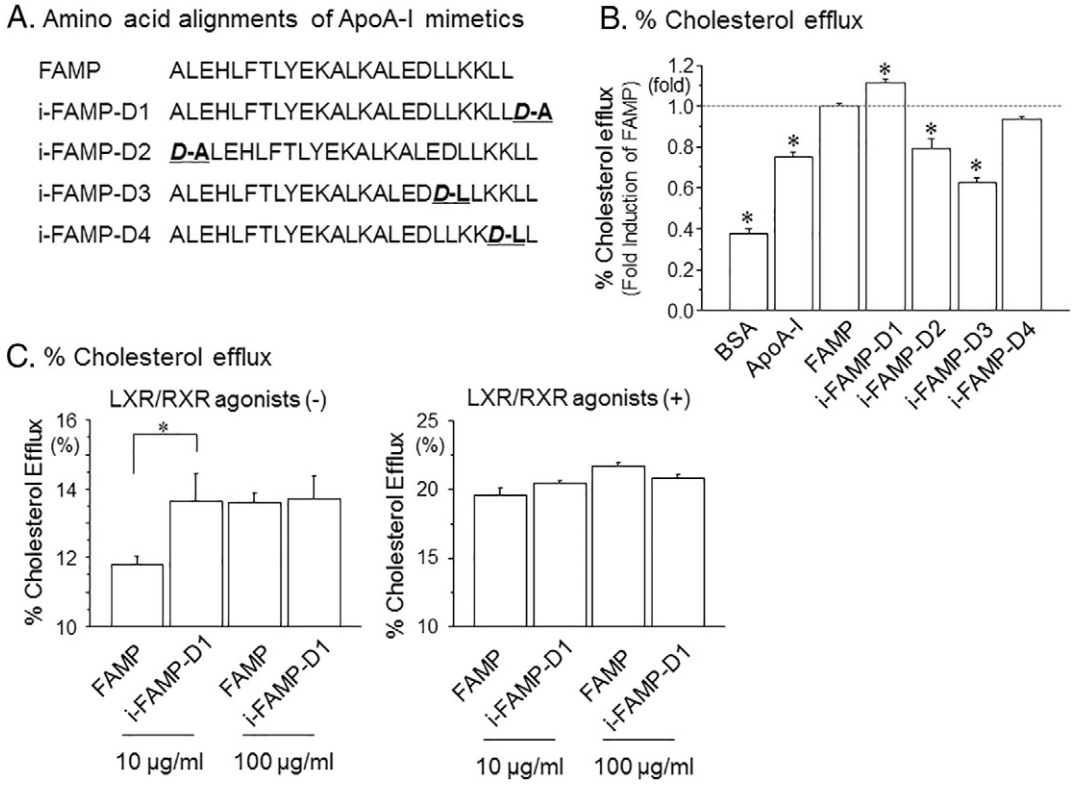

Fig. 1. A. Amino acid alignment in ApoA-I mimetics. B. % Cholesterol efflux using BSA, ApoA-I, FAMP and i-FAMPs. A172 cells were stimulated with 5 μmol/L of T0901317 and 9-cis-retinoic acid, and then 20 μg/ml of ApoA-I or each mimetic peptide was incubated with efflux medium. *$p < 0.01$ vs. FAMP ($n = 9$ each group). C. % Cholesterol efflux with 2 doses (10 μg/ml and 100 μg/ml) of FAMP or iFAMP-D1 incubated with plasma at 37 °C in vitro. A172 cells were stimulated with or without 5 μmol/L of T0901317 and 9-cis-retinoic acid, which are LXR/RXR agonists, and then treated with each peptide was incubated with efflux medium. *$p < 0.01$ ($n = 4$ in each group).

2.6. Lipoprotein analyses by agarose gel electrophoresis and ApoA-I immunoblotting

HDL samples were incubated with FAMP, i-FAMP-D1 or saline at 37 °C for 60 min. Agarose gel electrophoresis was performed using a Rapid Electrophoresis System (REP, Helena Laboratories) according to the method described previously [21]. After transfer to a PVDF membrane, ApoA-I was identified by immunoblotting with anti-ApoA-I antibody.

2.7. Mice

The study protocol was approved by the Animal Care and Use Committee of Fukuoka University. Mice were housed in specific pathogen-free barrier facilities at Fukuoka University and maintained under a 12-hour light/dark cycle, fed a standard rodent diet (CLEA Japan at Nihon Bioresearch), and provided with water ad libitum except where noted.

2.8. Pharmacokinetics of FAMP and i-FAMP-D1 in C57BL6 mice

Seven-week-old male C57BL6 mice were purchased from KBT Oriental (Tokyo, Japan). C57BL6 mice (n = 3) were intravenously administered 10 mg/kg of fluorescence-labeled Acd-FAMP or Acd-i-FAMP-D1. Plasma fluorescence signals were measured 0, 10, 30, 60, 120, 180, 240, 360 and 480 min after injection.

2.9. Macrophage RCT study of FAMP and i-FAMP-D1 in CETP transgenic (CETP Tg) mice

The RCT study was performed as described previously [22,23]. J774 cells were grown and radiolabeled with ^{3}H-cholesterol and cholesterol enriched with Ac-LDL for 40 h. The labeled foam cells were washed, equilibrated in medium, spun down and resuspended in medium immediately before use. CETP Tg mice (n = 22) were fed a 0.5% high-cholesterol diet containing 10% coconut oil for 3 weeks before experiments as prefeeding. For this experiment, mice were divided into 3 groups: FAMP (50 mg/kg of body weight per day), i-FAMP-D1 (50 mg/kg of body weight per day) or saline treatment for 5 days. J774 macrophage cells radiolabeled with tritium-cholesterol were injected intraperitoneally at day 4. Plasma was collected at 4, 24 and 48 h after tritium injection and feces was collected continuously until 48 h. Samples were subjected to liquid scintillation counting.

2.10. Treatment of ApoE knockout (KO) mice with FAMP or i-FAMP-D1 and evaluation of aortic atherosclerotic lesion formation

Six-week-old male ApoE KO mice were purchased from KBT Oriental (Tokyo, Japan). ApoE KO mice (n = 18) fed a high-fat diet (0.5% cholesterol and 10% fat) were intraperitoneally administered either FAMP (50 mg/kg), i-FAMP-D1 (50 mg/kg) or placebo as a control 2 times per week for 16 weeks. After 16 weeks of treatment, whole aortas were collected, and the extracted aortic tissues were stained with Oil Red O. Oil Red O-stained plaque lesions were calculated using ImageJ 1.45 s

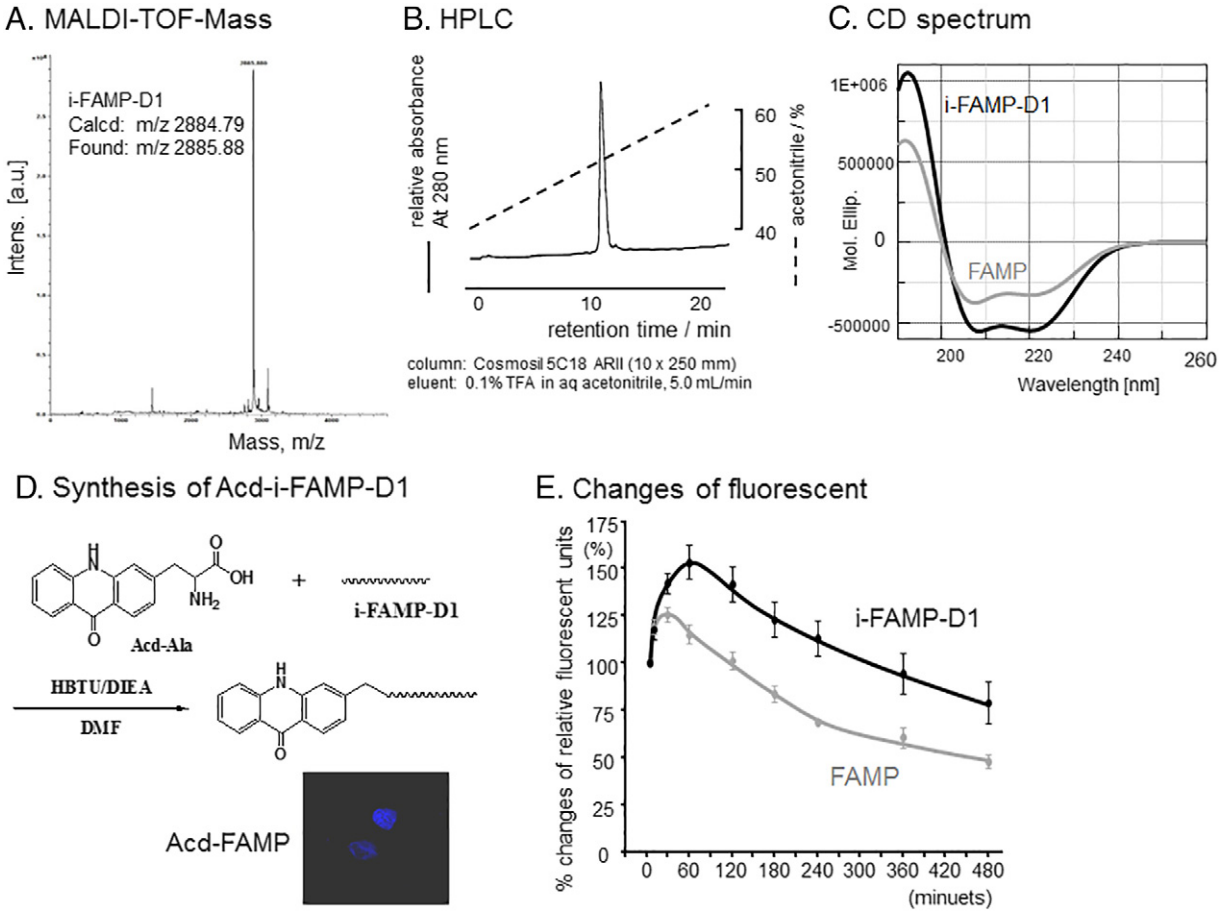

Fig. 2. Characteristics of iFAMP-D1 assessed by MALDI-TOF-MASS (A), HPLC (B) and CD spectra (C). D) Synthesis of Acd-FAMP. A representative image of blue fluorescence after incubation of A172 cells with Acd-FAMP. E) Pharmacokinetics of Acd-FAMP in C57BL6 mice.

software, and the extent of atherosclerosis was expressed as the percentage of the lesion area extending from the ascending aorta to the abdominal bifurcation.

2.11. Statistical analysis

All statistical analyses were performed using SigmaStat software (SYSTAT Software Inc., San Jose, CA). Differences between groups were analyzed by an unpaired *t*-test or one-way analysis of variance followed by Fisher's protected least significant difference test. All data are presented as the mean ± standard deviation. A probability value <0.05 was considered statistically significant.

3. Results

3.1. Amino acid alignments of ApoA-I mimetics and % cholesterol efflux

Amino acid alignments of ApoA-I mimetics are shown in Fig. 1A. Four candidate i-FAMPs were developed. D-alanine was added to the C-terminus and N-terminus ends of i-FAMP-D1 and i-FAMP-D2, respectively, to give FAMPs consisting of 25 amino acids. We examined which i-FAMP has greater cholesterol efflux capacity than conventional FAMP (Fig. 1B). Only i-FAMP-D1 showed a significantly stronger cholesterol efflux capacity than conventional FAMP. Thus, we selected i-FAMP-D1 for further experiments.

Two doses (10 μg/ml and 100 μg/ml) of FAMP or i-FAMP-D1 were incubated with plasma after 5 min of stimulation with or without 5 μM of T0901317 and 9-cis-retinoic acid, which are LXR/RXR agonists (Fig. 1C). % Cholesterol efflux was analyzed. i-FAMP-D1-mediated cholesterol efflux was significantly greater than FAMP-mediated efflux without LXR/RXR agonists. After stimulation with LXR/RXR agonists, FAMP-D1-mediated cholesterol efflux was increased, and specific cholesterol efflux with iFAMP-D1 was much higher than that with FAMP or ApoA-I (13.6% increase with i-FAMP, 57.1% increase with ApoA-I, $p < 0.01$). Furthermore, after incubation with i-FAMP-D1, the efflux capacity of HDL was significantly elevated.

3.2. Characteristics of i-FAMP-D1 as assessed by MALDI-TOF-mass, HPLC and CD spectra

The characteristics of i-FAMP-D1 after purification were determined by MALDI-TOF-Mass and HPLC (Fig. 2AB). The degree of purification of i-FAMP-D1 was found to be very high. CD spectra of i-FAMP-D1 and FAMP in water were also analyzed. i-FAMP-D1 formed stronger α-helical conformations than FAMP (Fig. 2C).

3.3. Synthesis of Acd-FAMP and pharmacokinetics of Acd-FAMP in C57BL6 mice

A representative picture of blue fluorescence after A172 cells were incubated with Acd-FAMP is shown in Fig. 2D. C57BL6 mice were intravenously administered 10 mg/kg of fluorescence-labeled Acd-FAMP or Acd-i-FAMP-D1 (Fig. 2E). Thirty minutes after Acd-i-FAMP-D1 injection, plasma fluorescence signals peaked, which was similar to the trend after Acd-FAMP injection. The fluorescence signals after Acd-i-FAMP-D1 injection were much stronger than those after Acd-FAMP injection throughout the study period.

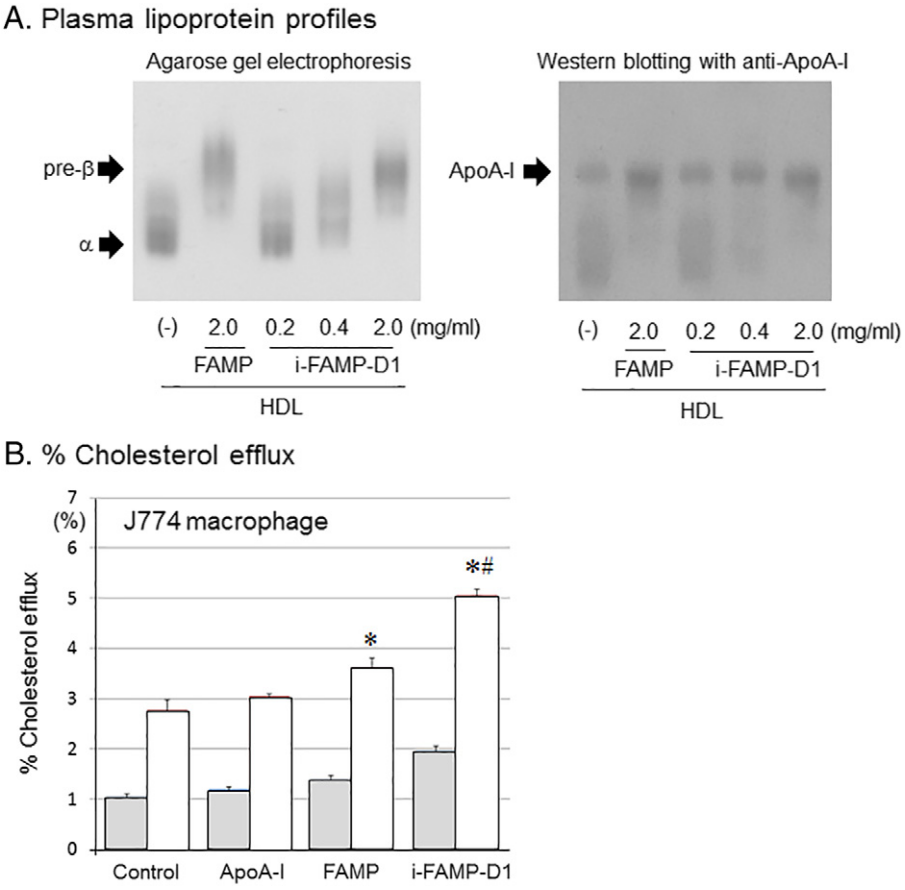

Fig. 3. A. Lipoprotein profile analyses by agarose gel electrophoresis and western blotting with anti-ApoA-I antibody. B. % Cholesterol efflux using placebo control, ApoA-I, FAMP and i-FAMPs in J774 macrophage cells. Gray and open bars indicate cAMP(−) and cAMP(+), respectively. *p < 0.05 vs. control, #p < 0.05 vs. FAMP.

3.4. Lipoprotein analyses by agarose gel electrophoresis and ApoA-I western blotting

Lipoprotein profile analyses by agarose gel electrophoresis and western blotting with anti-ApoA-I antibody are shown in Fig. 3A. Incubation with FAMP or i-FAMP-D1 shifted the α HDL band to the pre-β HDL position. Western blot analysis with anti-ApoA-I revealed ApoA-I at the pre-β HDL position even with a low dose of i-FAMP-D1. These observations suggest that incubation of HDL with FAMP or i-FAMP-D1 transformed α HDL to pre-β HDL. A high dose of FAMP or i-FAMP-D1 completely converted α HDL to pre-β HDL. % Cholesterol efflux using placebo control, ApoA-I, FAMP or i-FAMPs in J774 macrophage cells is shown in Fig. 3B. FAMP and i-FAMP-D1 significantly increased % Cholesterol efflux with cAMP stimulation, and i-FAMP-D1 showed a significantly stronger cholesterol efflux capacity with cAMP stimulation than FAMP.

3.5. Treatment of CETP Tg mice with FAMP or i-FAMP-D1 and measurement of ^{3}H-cholesterol in plasma and feces

Fig. 4AB shows the effects of FAMP and i-FAMP-D1 on RCT in CETP Tg mice. The i-FAMP-D1 group showed a statistically significant elevation of the amount of ^{3}H-cholesterol in feces compared to the FAMP and control groups, whereas there were no differences in the amount of ^{3}H-cholesterol in plasma among the 3 groups.

3.6. Treatment of ApoE KO mice with FAMP or i-FAMP-D1 and evaluation of aortic atherosclerotic lesion formation

After 16 weeks of treatment with FAMP, i-FAMP-D1 or saline, whole aortas were collected and the extracted aortic tissues were stained with Oil Red O. Representative pictures are shown in Fig. 4C. The relative surface areas of the plaque lesions in the i-FAMP-D1 group were significantly suppressed compared to those in the control and FAMP groups (Fig. 4D).

4. Discussion

In this study, we successfully developed i-FAMP, which had greater cholesterol efflux capacity than conventional FAMP. i-FAMP-D1 had a greater atheroprotective effect than FAMP in ApoE KO mice. It activated more ABCA1-dependent cholesterol efflux from macrophage cells to HDL than FAMP and increased cholesterol excretion from the liver to feces.

We synthesized a novel functional i-FAMP-D1 that consisted of 25 amino acids without phospholipids. Previously, FAMP has been shown to have anti-atherosclerotic and anti-inflammatory effects in vitro and in animal models [15]. We tried to create an ApoA-I mimetic peptide with stronger and longer-lasting effects than FAMP because mimetics are not yet available for clinical use. We considered that the degradation of FAMP by enzymes could be suppressed and its effect could last longer if we added D-alanine to the C-terminus or N-terminus of FAMP. The

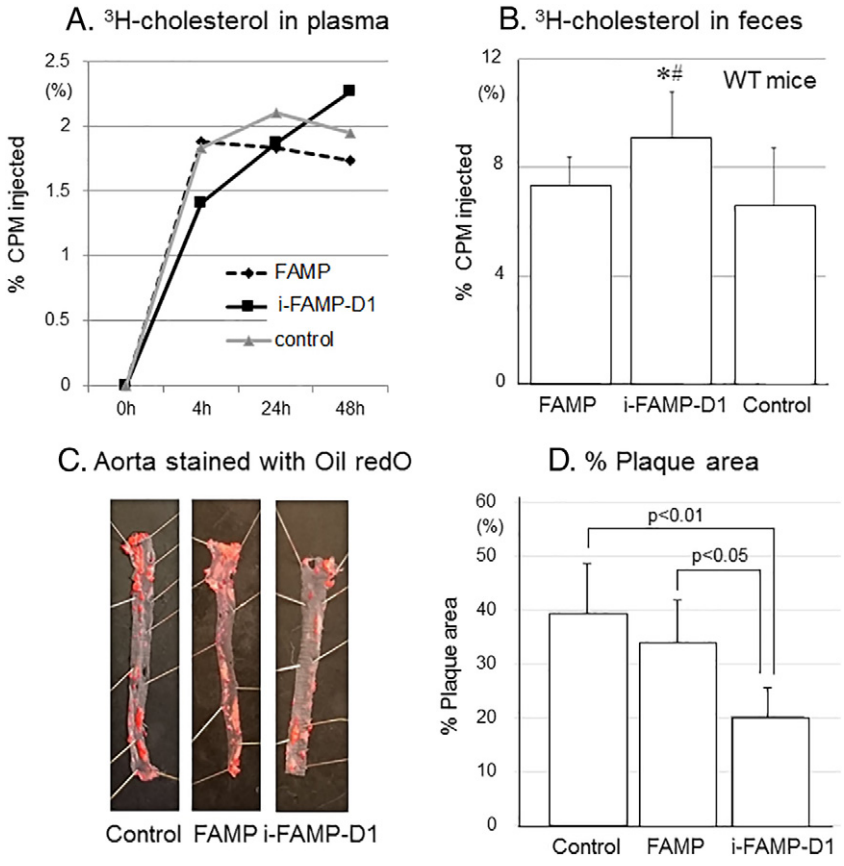

Fig. 4. Effects of FAMP and i-FAMP-D1 on RCT in CETP Tg mice. Mice were fed a high-cholesterol diet for 3 weeks, and then divided into FAMP (n = 7), i-FAMP-D1 (n = 8) and placebo control (n = 7) groups. The respective drugs were administered for 5 days and RCT was studied as described in the Methods. ^{3}H-cholesterol in plasma (A) and feces (B) after macrophage injection are shown. CPM, counts per minute. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. FAMP. Anti-arteriosclerotic effect in the aorta of ApoE KO mice. Suppressive effect of i-FAMP-D1 on atherosclerotic lesion formation in ApoE KO mice fed a high-fat diet. ApoE KO mice fed a high-fat diet (0.5% cholesterol and 10% fat) were intraperitoneally treated 3 times per week with FAMP (n = 6), i-FAMP-D1 (50 mg/kg body weight; n = 6), or placebo control (n = 6). After 16 weeks, whole aortas were collected and stained with Oil Red O (C). The extent of atherosclerosis is expressed as the percent of the lesion area extending from the ascending aorta to the abdominal bifurcation (D).

fluorescence signals after Acd-i-FAMP-D1 injection were much stronger than those after Acd-FAMP injection (Fig. 2E). In addition, i-FAMP-D1 formed a stronger α-helical conformation than FAMP (Fig. 2C). i-FAMP-D1 induced stronger cholesterol efflux capacity than FAMP. The induction of i-FAMP-D1-mediated cholesterol efflux in cells with LXR/RXR activation (Fig. 1C) strongly suggested that i-FAMP-D1 interacted with a membrane cholesterol transporter such as ATP-binding cassette transporter A1 (ABCA1). In fact, Xie et al. reported that the ApoA-1 mimetic D-4F promoted cholesterol efflux in macrophages through a cAMP-protein kinase ABCA1 pathway [24].

Pre-β HDL is a key molecule for functional HDL [25]. Incubation of HDL with a high dose of FAMP or i-FAMP-D1 transformed α HDL to pre-β HDL (Fig. 4A). The effect of i-FAMP-D1 was comparable to that of FAMP. We performed a macrophage RCT study of FAMP and i-FAMP-D1 in C57BL6 mice (Fig. 3C). RCT involves 3 critical steps: cholesterol efflux from macrophages to plasma HDL acceptors, HDL-C uptake from plasma to the liver, and HDL-derived cholesterol excretion from the liver to feces through bile. i-FAMP-D1 caused a significant increase in the amount of ^{3}H-cholesterol in feces compared to FAMP and controls, although there were no differences in the amount of ^{3}H-cholesterol in plasma among the 3 treatments. The third step might be increased in this experiment.

In this study, the anti-atherosclerotic effect of FAMP was weak compared to that in our previous study [15]. In the previous experiment, the area of aortic plaque with FAMP was about 17%, and the plaque area in the control group was about 32%. Thus, there was about a 15% difference in plaque area between the FAMP and control groups. On the other hand, in the present study, the plaque areas in the FAMP and control groups were about 33% and 39%, respectively (only a 6% difference). In this study, ApoE KO mice were intraperitoneally administered FAMP twice a week for 16 weeks. In the previous study, ApoE-deficient mice were administered FAMP 3 times a week. The plaque area with i-FAMP-D1 was about 20% (19% difference compared to the control), even though ApoE KO mice were administered i-FAMP-D1 twice a week. The anti-atherosclerotic effect of i-FAMP-D1 at twice a week (32 times/16 weeks) was comparable to that of FAMP at 3 times a week (48 times/16 weeks).

Therefore, in this study, i-FAMP-D1 had a greater atheroprotective effect than FAMP in ApoE KO mice due to its enhancement of RCT. The mechanism of enhancement by i-FAMP-D1 may be its greater cholesterol efflux capacity because it formed a stronger α-helical conformation and remained in the blood longer while transforming α HDL to pre-β HDL. In addition, inflammation plays a key role in the development of atherosclerosis [26,27]. CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome Study) provided the first evidence that targeting inflammation in humans with atherosclerosis could improve clinical outcomes [28]. ApoA-I mimetics also have anti-inflammatory effects in vitro and in animal models [15]. ApoA-I mimetics may enhance cholesterol efflux as well as having an anti-inflammatory effect. Although ApoA-I mimetics are not yet available for clinical use, our results are promising with regard to the prevention or regression of atherosclerosis. We have to make it possible to perform clinical research without limiting mimetic research to animal experiments.

Although we did not analyze the anti-obesity effect of i-FAMP in this study, ApoA-I mimetics have been reported to decrease adiposity [29,30]. The overexpression of ApoA-I and treatment with the mimetic D-4F remarkably reduced white fat mass and moderately improved insulin sensitivity. We need to analyze this effect using i-FAMP.

5. Conclusions

i-FAMP had a greater atheroprotective effect than FAMP in Apo-E knockout mice. In addition, i-FAMP activated more cholesterol efflux from macrophage cells to HDL than FAMP and increased cholesterol excretion from liver to feces.

Sources of funding

This work was supported by Grants-in-Aid for Scientific Research (Nos. 16K09484 and 19K08528) from the Ministry of Education, Culture, Sports, Science and Technology of Japan to K.S.

Declaration of competing interest

The authors report no relationships that could be construed as a conflict of interest.

Acknowledgments

We thank S. Abe and S. Tomita for their excellent technical assistance.

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