

## Original Article

# Beta-migrating very low-density lipoprotein conjugates with acrolein in high-cholesterol diet-fed rabbits and localizes to atherosclerotic lesions with macrophages

Yuri Kanogawa<sup>1</sup>, Masachika Fujiyoshi<sup>1</sup>, Yuki Nakazato<sup>1</sup>, Kenta Watanabe<sup>1</sup>, Mizuki Kurihara<sup>1</sup>, Akari Takezawa<sup>1</sup>, Masashi Uchida<sup>1</sup>, Kazuei Igarashi<sup>2,3</sup>, Takaaki Suzuki<sup>4</sup>, Noritaka Ariyoshi<sup>4</sup>, Itsuko Ishii<sup>1,4</sup>

Departments of <sup>1</sup>Clinical Pharmacy, <sup>2</sup>Clinical and Analytical Biochemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan; <sup>3</sup>Amine Pharma Research Institute, Innovation Plaza at Chiba University, Chiba, Japan; <sup>4</sup>Division of Pharmacy, Chiba University Hospital, Chiba, Japan

Received August 16, 2016; Accepted August 26, 2016; Epub November 1, 2016; Published November 15, 2016

**Abstract:** Protein-conjugated acrolein (PC-Acro) is detected in atherosclerotic lesions, and we demonstrated previously that acrolein-conjugated low-density lipoproteins induce macrophage foam cell formation. Although it has been suggested that  $\beta$ -migrating very low-density lipoprotein ( $\beta$ VLDL) is taken up by macrophages during atherogenesis, the modification of  $\beta$ VLDL with acrolein and its localization on lesions are still unclear. The purpose of this study was to clarify the localization of PC-Acro in atherosclerotic lesions and to determine the role of acrolein-conjugated  $\beta$ VLDL in atherogenesis. Male New Zealand white rabbits were fed 0.5% cholesterol-containing rabbit chow for 8 weeks, and used as an animal model of atherosclerosis. PC-Acro and malondialdehyde (MDA)-conjugated protein levels, which has been used widely as a means to detect oxidized low-density lipoprotein (LDL), in plasma were increased in the 0.5% cholesterol-containing diet-induced animal model of atherosclerosis, whereas their level was unchanged in the control diet fed rabbit. PC-Acro was detected in  $\beta$ VLDL by western blot analysis, and acrolein-conjugated  $\beta$ VLDL was effectively taken up by THP-1 macrophages. By immunohistochemical analysis, PC-Acro and macrophages were detected at the internal elastic lamina of the aorta, which was the initial lesion of atherosclerosis. These results suggest that acrolein-conjugated  $\beta$ VLDL has an important role in the initiation of atherosclerosis via the induction of macrophage foam cell formation in the atherosclerotic lesion.

**Keywords:** Acrolein, atherosclerosis, cholesterol, beta-migrating very low-density lipoprotein, macrophage

## Introduction

Cholesterol accumulation in macrophages with foam cell formation is the major cause of atherosclerosis. Since chemically oxidized low-density lipoprotein (LDL) is readily taken up by macrophages via scavenger receptors, in contrast to native LDL, oxidative modification of LDL has been widely believed to play a major role in cholesterol accumulation in macrophages [1]. Conversely, there is a controversial report that LDL isolated from the human aorta is not oxidized sufficiently to be taken up by macrophages via scavenger receptors [2]. Recently, we demonstrated that acrolein-conjugated LDL was taken up by macrophages more effectively than was oxidized LDL [3]. Therefore, it is possible that acrolein-conjugated lipoproteins play an important role in the

onset of atherosclerosis via the induction of macrophage foam cell formation.

Dyslipidemia is a risk factor for atherosclerosis [4]. The high-cholesterol diet-fed rabbit is a well characterized animal model of atherosclerosis, in which cholesteryl ester-rich lipoproteins, including  $\beta$ -migrating very low-density lipoprotein ( $\beta$ VLDL), are elevated in plasma [5, 6]. In humans, plasma  $\beta$ VLDL levels are elevated in type III hyperlipoproteinemia, which is characterized by the development of premature atherosclerosis [7]. Macrophages reportedly take up  $\beta$ VLDL and are transformed into foam cells in vitro [8-10]. Moreover, the plasma concentration of oxidized LDL is reportedly less than 0.5% of total LDL, which is not sufficient for proatherogenic activities [11]. These reports imply that  $\beta$ VLDL, but not oxidized LDL, is the

## Acrolein-beta VLDL induce atherosclerosis

major contributor to cholesterol accumulation in macrophages, and modified  $\beta$ VLDL affects the progression of atherosclerosis.

Acrolein is a toxic and highly reactive alpha, beta-unsaturated aldehyde generated mainly from unsaturated fatty acids by lipoxidation [12]. Protein-conjugated acrolein (PC-Acro) was presented in human atherosclerotic lesions [13], and the plasma levels of PC-Acro reportedly correlate with the development of carotid atherosclerosis in humans [14]. Acrolein is derived from two pathways: one is from overheated organic matter, such as cigarette smoking [15], which is a risk factor for coronary atherosclerosis; the other is from polyamines released from damaged cells, which is observed in a mouse model of brain infarction [16]. Nowadays, PC-Acro is used as a biochemical marker of brain infarction [17]. In addition, brain infarction is also strongly correlated with atherosclerosis [18]. From these observations, it is expected that  $\beta$ VLDL might be modified with acrolein, but the modification of  $\beta$ VLDL with acrolein and their impact on the initiation of atherosclerosis are unclear.

In this study, we examined the localization of PC-Acro and macrophages in an animal model of high-cholesterol diet-induced atherosclerosis. We further investigated the plasma concentrations of acrolein and malondialdehyde (MDA), which has been used widely as a means to detect oxidized LDL, to clarify the contribution of acrolein-conjugated  $\beta$ VLDL to atherogenesis. Moreover, we investigated the effect of acrolein conjugation on cholesterol accumulation by  $\beta$ VLDL in THP-1 macrophages.

### Materials and methods

#### *Ethics statement*

Written informed consent was given by each subject, and the study protocol was approved by the ethics committees of the Graduate School of Pharmaceutical Science, Chiba University. The investigations using animals described in this report was approved by the Chiba University Institutional Animal Care and Use Committee (Permit number, dou 26-190, dou27-248).

#### *Rabbits and food*

Male New Zealand white rabbits (6 weeks of age, 1.8-2.2 kg) were obtained from Takasugi Experimental Animal Supply Co., Ltd. (Saitama,

Japan). The rabbits were divided randomly into two groups. The control diet group (n = 5) was fed rabbit chow (RC) (normal rabbit chow; Oriental Yeast Co., Ltd., Tokyo, Japan), while the high-cholesterol diet group (n = 5) was fed 0.5% cholesterol-containing RC prepared by Oriental Yeast Co., Ltd. The rabbits were fed a 100 g diet every day for 8 weeks, and blood was collected every 2 weeks from the posterior auricular artery.

#### *Reagents*

Acrolein was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were commercial products of reagent grade.

#### *Determination of PC-Acro and cholesterol levels*

PC-Acro levels were determined using the ACR-Lysine Adduct ELISA System (NOF Corporation, Tokyo, Japan) according to the manufacturer's protocol. Total cholesterol and free cholesterol levels were measured using a Cholesterol E-test Wako Kit and Free Cholesterol E-test Wako Kit (Wako Pure Chemical Industries, Osaka, Japan), respectively. High-density lipoprotein (HDL) cholesterol levels were measured using the supernatant from the precipitate of non-HDL lipoproteins with phosphotungstic acid and magnesium chloride. Non-HDL cholesterol levels were calculated as the HDL cholesterol levels subtracted from the total cholesterol levels.

#### *Determination of MDA levels*

MDA levels were determined as described previously [19]. For each determination, 20  $\mu$ L of 20% trichloroacetic acid were added to a 60  $\mu$ L sample, and the samples were incubated for 10 min at 4°C. After centrifugation at 16,100 $\times$ g for 20 min at 4°C, 60  $\mu$ L of the supernatant were added to 10  $\mu$ L of 0.1125 M HClO<sub>4</sub> and 10  $\mu$ L of 40 mM 2-thiobarbituric acid (Sigma Chemical Co., St Louis, MO, USA) (final 14.1 mM HClO<sub>4</sub>, 5 mM 2-thiobarbituric acid). The reaction mixture was incubated at 94°C for 60 min, and absorbance at 532 nm was measured. MDA standards were prepared from the hydrolysis of 1, 1, 3, 3-tetraethoxypropane (Sigma Chemical Co.).

#### *Western blot analysis*

Blood samples were collected after feeding for 8 weeks. The rabbits were fasted for 1 day

## Acrolein-beta VLDL induce atherosclerosis

before blood collection, and were anesthetized with an injection of pentobarbital (50 mg/kg, i.p.). To prepare plasma, blood was drawn from the carotid artery and collected into sterile plastic tubes containing heparin (1 U/mL blood). After centrifugation at 1,000×g for 20 min at 4°C, plasma was obtained from the supernatant.  $\beta$ VLDL (density < 1.006 g/mL) was prepared from the plasma of the high-cholesterol diet group as described previously [20]. Very low-density lipoprotein (VLDL) and LDL were isolated from human plasma by sequential ultracentrifugation [20].  $\beta$  mobility was confirmed by agarose gel electrophoresis in barbital buffer solution using universalgel/8 (Helena Laboratories, Beaumont, TX, USA) with Fat Red 7B staining (MP Biomedicals, Santa Ana, CA, USA).

Acrolein-conjugated LDL (Acro-LDL), used as a control, was prepared by incubation of human LDL and acrolein at 37°C for 24 hr. Protein concentration was determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. The proteins were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and subsequently electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, CA, USA). The membranes were treated with a blocking buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 5% skimmed milk) and incubated with an anti-Acro-Lys (1:1,000; mAb5F6, NOF Corporation, Tokyo, Japan), anti-apolipoprotein B (apoB) (1:500; Santa Cruz Biotech, Inc., CA, USA), or anti-MDA antibody (1:1,000; NOF Corporation) for 3 hr at room temperature as the primary antibody. The membranes were washed with the blocking buffer and incubated with a horseradish peroxidase-conjugated second antibody. Immunoreactivity was visualized with Immobilon Western (Millipore) or ECL Western Blotting Detection System (GE Healthcare, Germany). The relative densities of the bands were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### *Determination of cellular cholesterol content*

$\beta$ VLDL (0.4 mg protein/mL) was incubated with indicated concentration of acrolein at 37°C for 24 hr under nitrogen gas, and then dialyzed against 0.9% NaCl and RPMI-1640 medium

(RPMI, Wako Pure Chemical Industries). The mobility of the lipoproteins in agarose gel electrophoresis was checked as described above. After filtrating with surfactant-free cellulose acetate membrane (pore size 0.45  $\mu$ m), acrolein-conjugated  $\beta$ VLDL was used for cholesterol accumulation study.

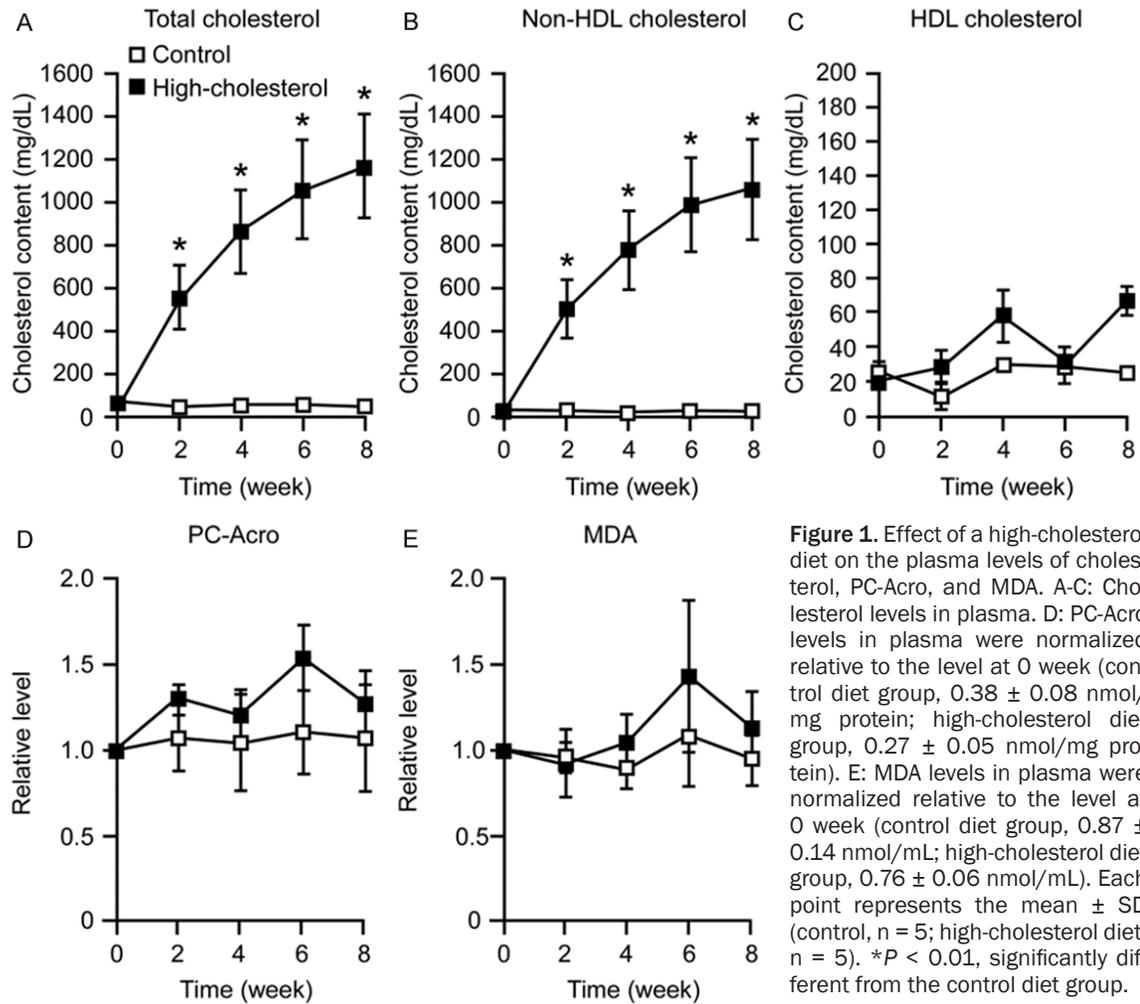
Human acute monocytic leukemia (THP-1) cells (American Type of Cell Culture, Rockville, MD, USA) were maintained in RPMI supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin and 10% fetal bovine serum (FBS). THP-1 cells ( $1 \times 10^6$  cells/mL) were treated with 150 nM phorbol 12-myristate 13-acetate (PMA) for 48 hr in RPMI supplemented with 10% FBS, and the adherent cells were used as macrophages (THP-1 macrophages).

For cholesterol accumulation study, THP-1 macrophages were seeded at a density of  $1 \times 10^6$  cells/well on 12-well culture plates (Costar, Cambridge, MA, USA). THP-1 macrophages were treated with 1 mL of RPMI supplemented with 2% FBS and 0.2 mg cholesterol/mL acrolein-conjugated  $\beta$ VLDL for 48 hr. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) supplemented with 0.1% BSA, twice with ice-cold PBS, and then treated with 1 mL hexane/isopropanol (2:1, volume/volume). Total and free cholesterol levels were determined in the same way as described above. Cholesteryl ester level was calculated as the free cholesterol level subtracted from the total cholesterol level.

### *Immunohistochemical analysis*

Aortas were perfused in situ with PBS followed by 4% paraformaldehyde, and they were then immersed in PBS containing graded sucrose (10%, 20%, 30%) for 16 hr at 4°C. The aorta was divided into four segments, as described previously [21]: the ascending aortic arch; the downstream region of the left subclavian artery; the aorta of the intermediate region between the left subclavian artery and diaphragm; and the abdominal aorta under the diaphragm. Frozen sections (5  $\mu$ m thickness) were made using a cryostat (CM1850; Leica, Heidelberg, Germany) and then mounted on silanized glass slides. Frozen sections of the aortic arch were used for immunohistochemistry. Brown staining using the chromogen diaminobenzidine was performed using a VECTASTAIN Elite ABC Kit

## Acrolein-beta VLDL induce atherosclerosis



(Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. An anti-rabbit macrophage (1:1,000; RAM11, Dako North America, Inc., Carpinteria, CA, USA), anti-Acro-Lys (1:1,000), anti- $\alpha$ -muscle actin (1:200; HHF35, Dako North America, Inc.), or anti-CD31 antibody (1:100; JC07A, Dako North America, Inc.) was used as the primary antibody. The frozen sections were pre-incubated with 10% normal goat serum (Wako Pure Chemical Industries) for 10 min, and then incubated with the primary antibody for 16 hr at 4°C. All sections were counterstained with hematoxylin, blued in Scott's tap water, dehydrated, and mounted using CC/Mount (Diagnostic Biosystems, Inc., Pleasanton, CA, USA).

### Data analysis

Unless otherwise indicated, all data represent the mean  $\pm$  standard deviation (SD) values. An unpaired, two-tailed Student's t-test was used

to determine the significance of differences between two groups means, taking  $P < 0.01$  as the criterion of a significant difference between two group means. The statistical significance of differences among means of more than two groups was determined by a one-way ANOVA followed by the Bonferroni Multiple Comparisons Test, taking  $P < 0.01$  as the criterion of a significant difference between two group means.

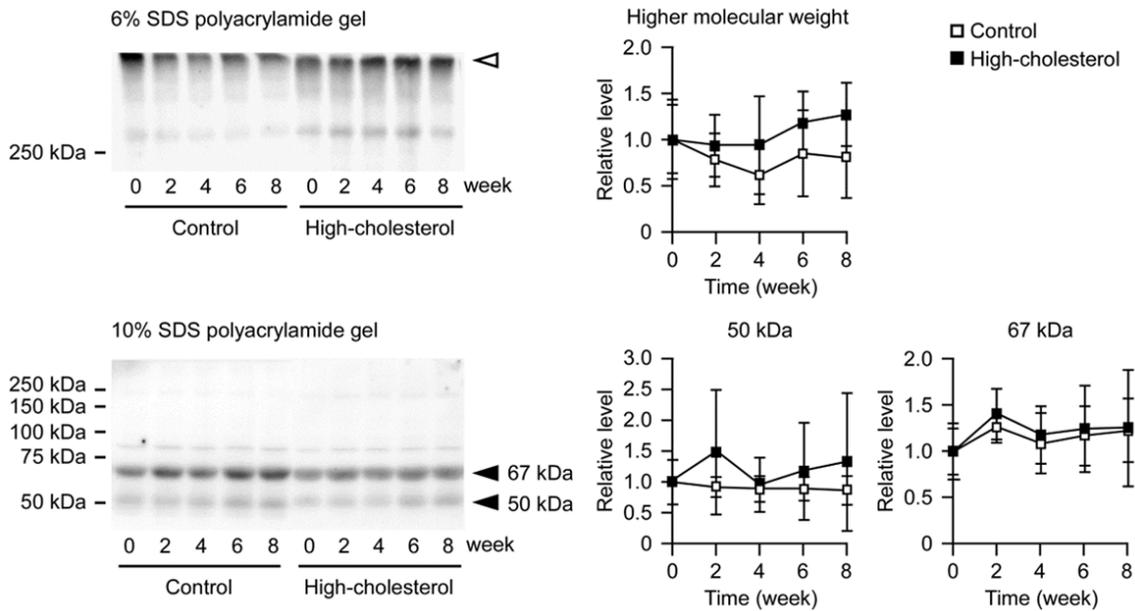
### Results

#### Effect of the high-cholesterol diet on the plasma levels of cholesterol, PC-Acro, and MDA in rabbits

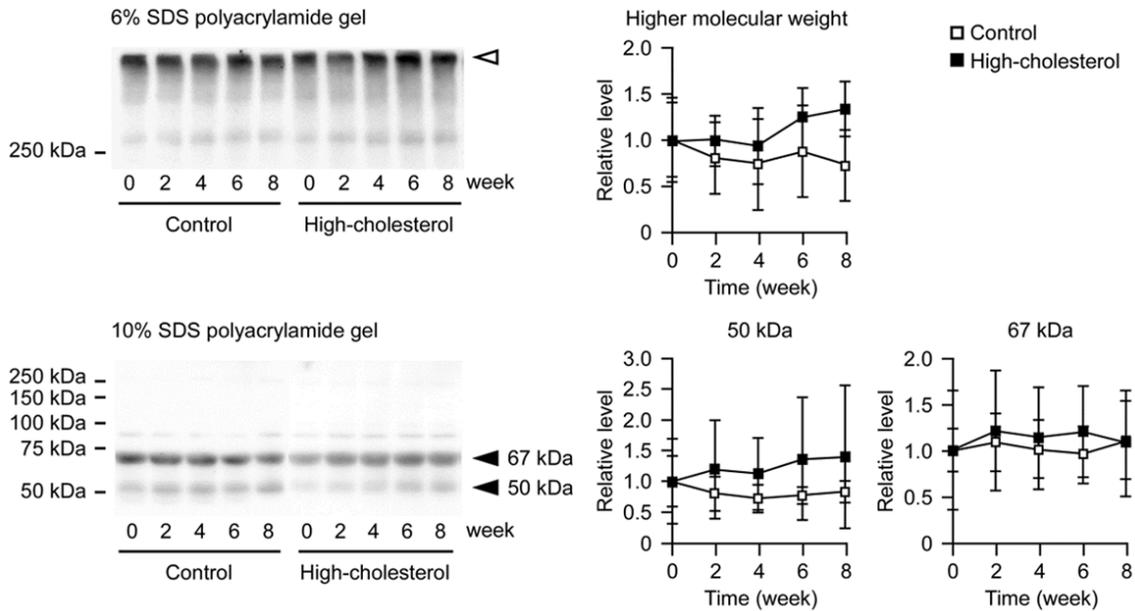
There was no significant difference in body weight between the two groups. The initial body weight of the control and high-cholesterol diet groups was  $1.94 \pm 0.15$  kg and  $1.94 \pm 0.16$  kg, respectively; body weight at the end of the

# Acrolein-beta VLDL induce atherosclerosis

## A PC-Acro



## B MDA



**Figure 2.** Effect of a high-cholesterol diet on PC-Acro (A) and MDA-conjugated protein levels (B) in rabbit plasma. Total protein (30  $\mu$ g) was subjected to SDS-PAGE with a 6% or 10% polyacrylamide gel. Representative patterns of western blot analysis are shown. Band intensity was normalized relative to the intensity at 0 week. Each point represents the mean  $\pm$  SD (control diet group, n = 5; high-cholesterol diet group, n = 5). The white arrowhead indicates the expected size of apoB cross-linking with acrolein or MDA, and described as "higher molecular weight".

experiment was  $2.55 \pm 0.09$  kg and  $2.70 \pm 0.19$  kg, respectively. Total plasma and non-HDL cholesterol levels were significantly increased in the high-cholesterol diet group (**Figure 1A, 1B**), while HDL cholesterol levels were unchanged (**Figure 1C**). PC-Acro and MDA plas-

ma levels in the control diet group were unchanged during the 8 week experimental period. Plasma PC-Acro levels were increased from 2 weeks after the onset of the high-cholesterol diet, and the highest level was observed at 6 weeks (**Figure 1D**). Plasma MDA le-

## Acrolein-beta VLDL induce atherosclerosis

vels were increased from 4 weeks after the onset of the high-cholesterol diet, and the highest level was observed at 6 weeks (**Figure 1E**). The majority of PC-Acro and MDA values in the high-cholesterol diet group were higher than in the control diet group, but there was no statistically significant difference between the two groups.

### *Effect of the high-cholesterol diet on the profile of PC-Acro and MDA-conjugated protein in rabbit plasma*

The expressional profiles of PC-Acro and MDA-conjugated protein in plasma were examined by western blot analysis. The intensity of 67 kDa bands, which was corresponding to albumin conjugated with acrolein or MDA, was unchanged during the 8 weeks of the experimental period (**Figure 2A, 2B**; black arrowheads). The unidentified proteins whose molecular size is around 50 kDa were also unchanged during the experimental period (**Figure 2A, 2B**; black arrowheads). The intensity of the high-molecular-weight band, which is the expected size of apoB cross-linking with acrolein or MDA, was increased in the high-cholesterol diet group from 6 weeks (**Figure 2A, 2B**; white arrowhead), but there was no statistically significant difference between the two groups.

### *Detection of acrolein- and MDA-conjugated protein in rabbit $\beta$ VLDL*

The bands at ~500 kDa (black arrowhead) or more than 1,000 kDa (white arrowhead) were detected strongly in  $\beta$ VLDL with Coomassie brilliant blue (CBB) staining and with the anti-apoB antibody (**Figure 3A, 3B**, respectively). Using the anti-Acro-Lys or anti-MDA antibody, the bands were detected at the same molecular weight as those detected by the anti-apoB antibody (**Figure 3D, 3E**, respectively).

The bands corresponding to IgG was presented in  $\beta$ VLDL fraction and mainly present in the largest band (**Figure 3C**). Recently, we identified the position of acrolein-conjugated amino acid residues in immunoglobulins [22], suggesting IgG, at least in part, was modified by acrolein and cross-linked with apoB in  $\beta$ VLDL.

### *Cholesterol accumulation in THP-1 macrophage by acrolein-conjugate $\beta$ VLDL*

By agarose gel electrophoresis, the mobility of  $\beta$ VLDL was smaller than that of human VLDL and larger than that of human LDL (**Figure 4A**), indicating  $\beta$ VLDL used in this study was  $\beta$  mobil-

ity. The mobility of  $\beta$ VLDL in agarose gel electrophoresis was increased by the treatment with acrolein in an acrolein-concentration dependent manner (**Figure 4A**). Total cholesterol, free cholesterol and cholesteryl ester accumulation in THP-1 macrophages by 2 mM acrolein-treated  $\beta$ VLDL was 1.9-, 1.6- and 2.4-fold greater than that by  $\beta$ VLDL, respectively (**Figure 4B**).

### *Localization of macrophages and PC-Acro in the aorta with or without atherosclerosis*

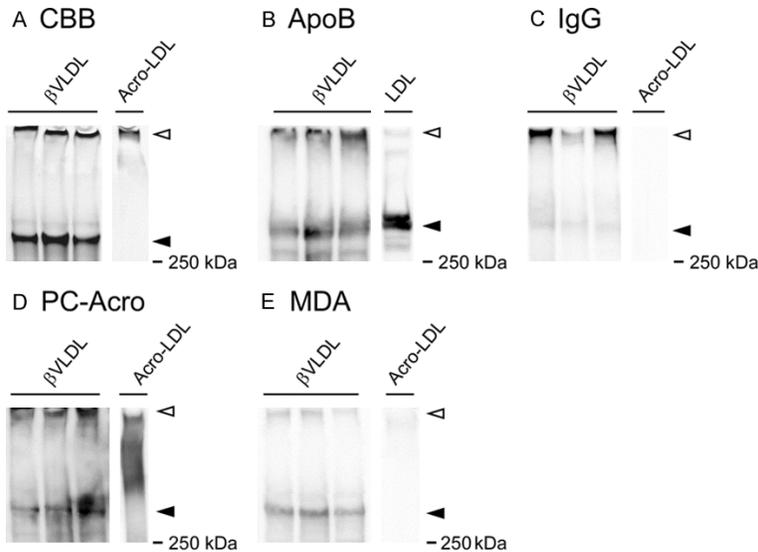
In the aortic arch of the control diet group, no signal was detected by using the anti-rabbit macrophage antibody (**Figure 5B**). PC-Acro was observed on the luminal side of the aortic arch (**Figure 5F**). The tunica media was positive for smooth muscle cells (SMC) (**Figure 5J**). Positive signals for endothelial cells were observed in the luminal side of the aortic arch (**Figure 5N**).

Atherosclerotic lesions were well developed in the high-cholesterol diet group. Macrophages were abundant in the atherosclerotic lesion of the aortic arch (**Figure 5D**). Positive signals for endothelial cells were observed in the luminal side of the aortic arch (**Figure 5P**). In the thickened intima of the atherosclerotic lesion, PC-Acro was observed on the internal elastic lamina in addition to the luminal side of the aortic arch (**Figure 5H**), and SMC were abundant on the lumen side (**Figure 5L**). Such characteristic signal was not seen without using primary antibody (**Figure 5A, 5C, 5E, 5G, 5I, 5K, 5M, 5O**).

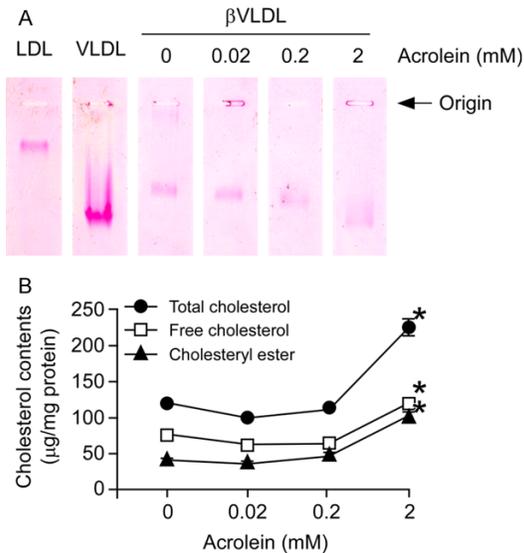
## Discussion

The present study demonstrates that PC-Acro and macrophages were localized in the atherosclerotic lesions of high-cholesterol diet-fed rabbits (**Figure 5D, 5H**). In the high-cholesterol diet-fed rabbits, cholesterol levels were significantly raised in the non-HDL fraction containing  $\beta$ VLDL (**Figure 1A-C**). The plasma concentration of PC-Acro was increased from 2 weeks after the initiation of the high-cholesterol diet (**Figure 1D**). By western blot analysis, it is suggested that apoB in  $\beta$ VLDL, which was observed in the high-cholesterol diet-fed animals, was conjugated with acrolein (**Figure 3**).  $\beta$ VLDL reportedly appears to be taken up by macrophages and is involved in the progression of atherosclerosis [8-10]. We demonstrated that THP-1 macrophages took up acrolein-conjugated  $\beta$ VLDL effectively (**Figure 4B**). Together with the present study,  $\beta$ VLDL conjugated with acrolein is suggested to play an important role in

## Acrolein-beta VLDL induce atherosclerosis



**Figure 3.** Detection of PC-Acro and MDA-conjugated protein in rabbit  $\beta$ VLDL. Total protein (30  $\mu$ g) was subjected to SDS-PAGE with a 3-10% gradient polyacrylamide gel. A: CBB staining after SDS-PAGE. B: Detection of apoB. C: Detection of IgG. D: Detection of PC-Acro. E: Detection of MDA. The black arrowhead indicates the expected size of apoB. The white arrowhead indicates the expected size of apoB cross-linking with acrolein.



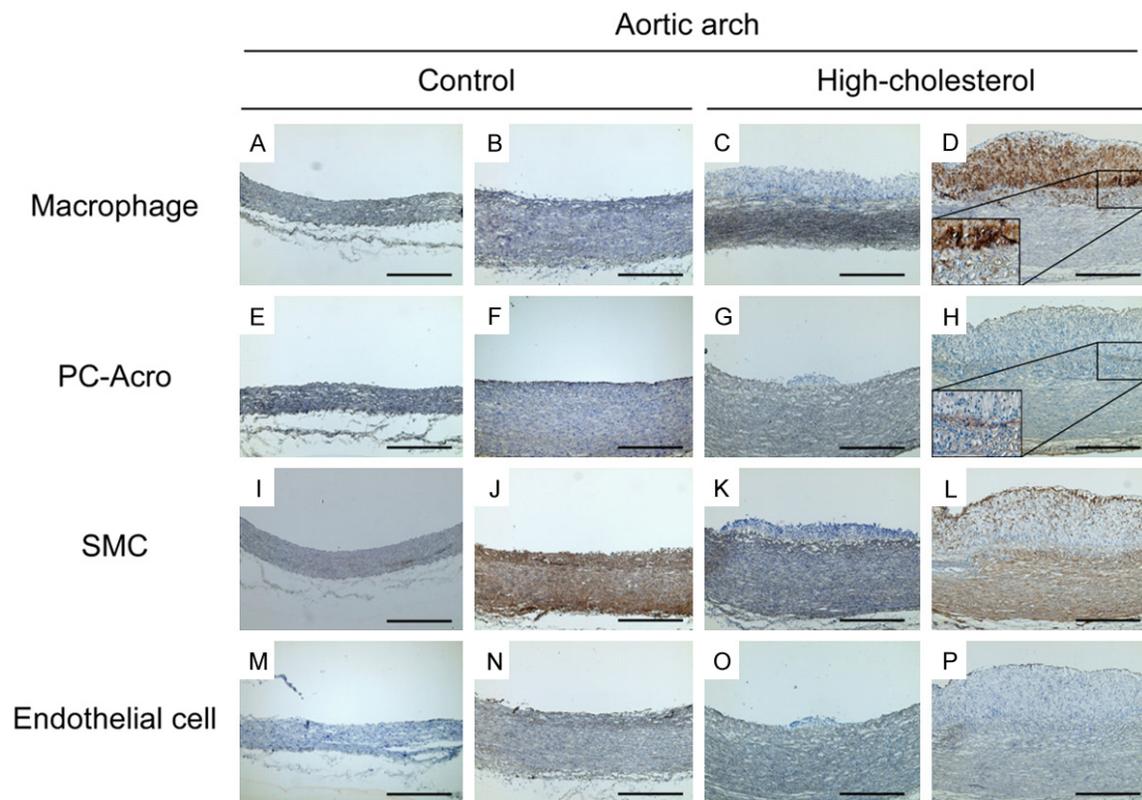
**Figure 4.** Cholesterol accumulation in THP-1 macrophages induced by acrolein-conjugated  $\beta$ VLDL. A: Agarose gel electrophoresis of LDL, VLDL and acrolein-conjugated  $\beta$ VLDL. Acrolein-conjugated  $\beta$ VLDL was prepared by using the indicated concentration of acrolein. B: Cholesterol contents of THP-1 macrophages incubated with 0.2 mg cholesterol/mL acrolein-conjugated  $\beta$ VLDL for 48 hr. Each point represents the mean  $\pm$  SD ( $n = 3$ ), and error bars smaller than the symbols are not shown. \* $P < 0.01$ , significantly different from the control.

the development of atherosclerosis.

The uptake of oxidized LDL, such as MDA-conjugated LDL, is thought to be a major factor in the formation of foam cells [1]. In contrast, Nakajima et al. raised the possibility that oxidized LDL has a minor role in the initiation of atherosclerosis, and oxidized LDL in atherosclerotic lesions was an end product that originated from native LDL from the circulatory system [11]. In this study, PC-Acro in rabbit was induced by high-cholesterol diet fed in the same manner as MDA-conjugated protein (Figure 2). It has been reported that antioxidant vitamins, such as vitamins E and B, do not reduce cardiovascular events [23, 24]. Moreover, acrolein-conjugated  $\beta$ VLDL was taken up effectively by macrophage (Figure 4B). Therefore, it is possible that acrolein-conjugated lipoproteins, such as  $\beta$ VLDL and LDL, have an important role in the development of atherosclerosis.

PC-Acro was observed on the blood side of endothelial cells of rabbits, regardless of whether they had been a high-cholesterol diet (Figure 5F, 5H). The production of acrolein derived from polyamines by polyamine oxidase is increased during cell damage in conditions such as stroke [25]. The luminal side of the endothelial cells is in direct contact with the blood flow, and is stimulated continuously by flow-induced shear stress [26]. Therefore, it is conceivable that acrolein was produced in endothelial cells via shear stress-induced cell damage. In the thickened intima, PC-Acro was restrictively localized at the internal elastic lamina of the aorta (Figure 5H), raising the possibility that PC-Acro play a role in the initiation of atherosclerosis. Acrolein-conjugated  $\beta$ VLDL was effectively taken up by THP-1 macrophage (Figure 4B), which leads to foam cell formation. So,  $\beta$ VLDL conjugated with acrolein was candidate molecule which localized in the initiation site of atherosclerosis.

PC-Acro was observed on the blood side of endothelial cells of rabbits, regardless of whether they had been a high-cholesterol diet (Figure 5F, 5H). The production of acrolein derived from polyamines by polyamine oxidase is increased during cell damage in conditions such as stroke [25]. The luminal side of the endothelial cells is in direct contact with the blood flow, and is stimulated continuously by flow-induced shear stress [26]. Therefore, it is conceivable that acrolein was produced in endothelial cells via shear stress-induced cell damage. In the thickened intima, PC-Acro was restrictively localized at the internal elastic lamina of the aorta (Figure 5H), raising the possibility that PC-Acro play a role in the initiation of atherosclerosis. Acrolein-conjugated  $\beta$ VLDL was effectively taken up by THP-1 macrophage (Figure 4B), which leads to foam cell formation. So,  $\beta$ VLDL conjugated with acrolein was candidate molecule which localized in the initiation site of atherosclerosis.



**Figure 5.** Immunohistochemistry of the aortic arch in high-cholesterol diet rabbits. A, C, E, G, I, K, M, O: Immunostaining without primary antibody (negative control); B, D: Immunostaining using an anti-macrophage antibody (RAM11); F, H: Immunostaining using an anti-Acro-Lys antibody (mAb5F6); J, L: Immunostaining using an anti- $\alpha$ -muscle actin antibody (HHF35); N, P: Immunostaining using an anti-CD31 antibody (JC07A). A, B, E, F, I, J, M, N: Control diet group; C, D, G, H, K, L, O, P: High-cholesterol diet group. Brown area is positive immunostaining and blue area is nuclear staining produced with hematoxylin. Insets: enlarged image. Scale bars: 200  $\mu$ m.

In conclusion, our results indicate that PC-Acro was localized in atherosclerotic lesions with macrophage accumulation. In addition,  $\beta$ VLDL was conjugated with acrolein in an animal model of atherosclerosis, and  $\beta$ VLDL conjugated with acrolein was effectively taken up by THP-1 macrophage. Therefore, acrolein-conjugated  $\beta$ VLDL suggested to be involved in the initiation of atherosclerosis.

#### Acknowledgements

This study was supported in part by JSPS KAKENHI Grant Number 22590057.

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Itsuko Ishii, Division of Pharmacy, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku 260-8677, Chiba, Japan. Tel: +81-43-226-

2347; Fax: +81-43-226-2347; E-mail: iishii@faculty.chiba-u.jp

#### References

- [1] Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF and Freeman MW. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem* 2002; 277: 49982-49988.
- [2] Steinbrecher UP and Loughheed M. Scavenger receptor-independent stimulation of cholesterol esterification in macrophages by low density lipoprotein extracted from human aortic intima. *Arterioscler Thromb* 1992; 12: 608-625.
- [3] Watanabe K, Nakazato Y, Saiki R, Igarashi K, Kitada M and Ishii I. Acrolein-conjugated low-density lipoprotein induces macrophage foam cell formation. *Atherosclerosis* 2013; 227: 51-57.

## Acrolein-beta VLDL induce atherosclerosis

- [4] Libby P, Ridker PM and Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature* 2011; 473: 317-325.
- [5] Gordon GB, Bush DE and Weisman HF. Reduction of atherosclerosis by administration of dehydroepiandrosterone. A study in the hypercholesterolemic New Zealand white rabbit with aortic intimal injury. *J Clin Invest* 1988; 82: 712-720.
- [6] Ouchi Y, Tabata RE, Stergiopoulos K, Sato F, Hattori A and Orimo H. Effect of dietary magnesium on development of atherosclerosis in cholesterol-fed rabbits. *Arteriosclerosis* 1990; 10: 732-737.
- [7] Mahley RW, Huang Y and Rall SC Jr. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia). Questions, quandaries, and paradoxes. *J Lipid Res* 1999; 40: 1933-1949.
- [8] Goldstein JL, Ho YK, Brown MS, Innerarity TL and Mahley RW. Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine beta-very low density lipoproteins. *J Biol Chem* 1980; 255: 1839-1848.
- [9] Mahley RW, Innerarity TL, Brown MS, Ho YK and Goldstein JL. Cholesteryl ester synthesis in macrophages: stimulation by beta-very low density lipoproteins from cholesterol-fed animals of several species. *J Lipid Res* 1980; 21: 970-980.
- [10] Tabas I, Lim S, Xu XX and Maxfield FR. Endocytosed beta-VLDL and LDL are delivered to different intracellular vesicles in mouse peritoneal macrophages. *J Cell Biol* 1990; 111: 929-940.
- [11] Nakajima K, Nakano T and Tanaka A. The oxidative modification hypothesis of atherosclerosis: the comparison of atherogenic effects on oxidized LDL and remnant lipoproteins in plasma. *Clin Chim Acta* 2006; 367: 36-47.
- [12] Uchida K, Kanematsu M, Morimitsu Y, Osawa T, Noguchi N and Niki E. Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. *J Biol Chem* 1998; 273: 16058-16066.
- [13] Uchida K, Kanematsu M, Sakai K, Matsuda T, Hattori N, Mizuno Y, Suzuki D, Miyata T, Noguchi N, Niki E and Osawa T. Protein-bound acrolein: potential markers for oxidative stress. *Proc Natl Acad Sci U S A* 1998; 95: 4882-4887.
- [14] Yoshida M, Higashi K, Kobayashi E, Saeki N, Wakui K, Kusaka T, Takizawa H, Kashiwado K, Suzuki N, Fukuda K, Nakamura T, Watanabe S, Tada K, Machi Y, Mizoi M, Toida T, Kanzaki T, Tomitori H, Kashiwagi K and Igarashi K. Correlation between images of silent brain infarction, carotid atherosclerosis and white matter hyperintensity, and plasma levels of acrolein, IL-6 and CRP. *Atherosclerosis* 2010; 211: 475-479.
- [15] Dong JZ and Moldoveanu SC. Gas chromatography-mass spectrometry of carbonyl compounds in cigarette mainstream smoke after derivatization with 2,4-dinitrophenylhydrazine. *J Chromatogr A* 2004; 1027: 25-35.
- [16] Saiki R, Park H, Ishii I, Yoshida M, Nishimura K, Toida T, Tatsukawa H, Kojima S, Ikeguchi Y, Pegg AE, Kashiwagi K and Igarashi K. Brain infarction correlates more closely with acrolein than with reactive oxygen species. *Biochem Biophys Res Commun* 2011; 404: 1044-1049.
- [17] Igarashi K and Kashiwagi K. Protein-conjugated acrolein as a biochemical marker of brain infarction. *Mol Nutr Food Res* 2011; 55: 1332-1341.
- [18] O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL and Wolfson SK Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl J Med* 1999; 340: 14-22.
- [19] Carbonneau MA, Peuchant E, Sess D, Canioni P and Clerc M. Free and bound malondialdehyde measured as thiobarbituric acid adduct by HPLC in serum and plasma. *Clin Chem* 1991; 37: 1423-1429.
- [20] Havel RJ, Eder HA and Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955; 34: 1345-1353.
- [21] Uchida M, Ishii I, Inoue C, Akisato Y, Watanabe K, Hosoyama S, Toida T, Ariyoshi N and Kitada M. Kefiran reduces atherosclerosis in rabbits fed a high cholesterol diet. *J Atheroscler Thromb* 2010; 17: 980-988.
- [22] Hirose T, Saiki R, Uemura T, Suzuki T, Dohmae N, Ito S, Takahashi H, Ishii I, Toida T, Kashiwagi K and Igarashi K. Increase in acrolein-conjugated immunoglobulins in saliva from patients with primary Sjogren's syndrome. *Clin Chim Acta* 2015; 450: 184-189.
- [23] Lonn E, Bosch J, Yusuf S, Sheridan P, Pogue J, Arnold JM, Ross C, Arnold A, Sleight P, Probstfeld J, Dagenais GR; Hope and Investigators H-TT. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA* 2005; 293: 1338-1347.
- [24] Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfeld J, Fodor

## Acrolein-beta VLDL induce atherosclerosis

- G, Held C, Genest J Jr; Heart Outcomes Prevention Evaluation (HOPE) 2 Investigators. Homocysteine lowering with folic acid and B vitamins in vascular disease. *N Engl J Med* 2006; 354: 1567-1577.
- [25] Tomitori H, Usui T, Saeki N, Ueda S, Kase H, Nishimura K, Kashiwagi K and Igarashi K. Polyamine oxidase and acrolein as novel biochemical markers for diagnosis of cerebral stroke. *Stroke* 2005; 36: 2609-2613.
- [26] Tarbell JM, Shi ZD, Dunn J and Jo H. Fluid Mechanics, Arterial Disease, and Gene Expression. *Annu Rev Fluid Mech* 2014; 46: 591-614.