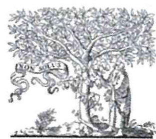
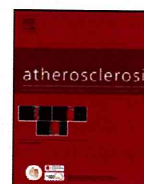
Atherosclerosis 218 (2011) 300–307



Contents lists available at ScienceDirect

Atherosclerosis

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



Antiatherogenic effects of newly developed apolipoprotein A-I mimetic peptide/phospholipid complexes against aortic plaque burden in Watanabe-heritable hyperlipidemic rabbits

Atsushi Iwata [a], Shin-ichiro Miura [a,*], Bo Zhang [b], Satoshi Imaizumi [a], Yoshinari Uehara [a], Masashi Shiomi [c], Keijiro Saku [a,*]

[a] Department of Cardiology, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-Ku, Fukuoka 814-0180, Japan
[b] Department of Biochemistry, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-Ku, Fukuoka 814-0180, Japan
[c] Institute for Experimental Animals, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-Ku, Kobe 650-0017, Japan

ARTICLE INFO

Article history:
Received 28 January 2011
Received in revised form 2 May 2011
Accepted 22 May 2011
Available online 1 June 2011

Keywords:
Apolipoprotein A-I mimetic peptide
Plaque
Cholesterol efflux
Watanabe-heritable hyperlipidemic rabbits
Intravascular ultrasound
Capillary isotachophoresis

ABSTRACT

This study analyzed the antiatherogenic effects of newly developed apolipoprotein A-I (ApoA-I) mimetic peptide/phospholipid complexes (ETC-642) against the aortic plaque burden in vivo. We used human macrophage cells to analyze cholesterol efflux by ETC-642. Watanabe-heritable hyperlipidemic (WHHL) rabbits were divided into 3 groups: low- (15 mg/kg) and high-dose ETC-642 (50 mg/kg), and placebo. The test material was injected twice/week for 12 weeks. The aortic plaque burden was assessed by intravascular ultrasound (IVUS) at 0 and 12 weeks. Plasma lipid profiles were analyzed by capillary isotachophoresis every 4 weeks. ETC-642 had an effect on cholesterol efflux comparable to that of conventional rHDL. In WHHL rabbits, high-dose ETC-642 inhibited the progression of aortic atherosclerosis compared to placebo. There was no change in the percentage of plaque volume (%PV) in the high-dose group between before (30.9%) and after infusion (28.6%), whereas there was a significant increase in the control group from 27.8% to 37.9%. ETC-642 significantly reduced charge-modified low-density lipoprotein (LDL) by converting more negative-charged modified LDL to less negative-charged LDL, and reduced small dense (sd) LDL by converting it into large, buoyant (lb) LDL. Changes in the %PV were positively correlated with changes in negative-charged modified LDL ($r = 0.61$, $p < 0.01$) and sdLDL ($r = 0.59$, $p < 0.01$), and negatively correlated with changes in less negative-charged LDL ($r = -0.43$, $p < 0.01$) and lbLDL ($r = -0.57$, $p < 0.01$). In conclusion, the ETC-642-induced remodeling of sdLDL to large and lbLDL and the enhancement of cholesterol efflux may prevent progression of the aortic plaque burden. HDL-based therapy may be useful for preventing the progression of plaque volume.

© 2011 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

A low serum high-density lipoprotein (HDL) cholesterol level is one of the strongest predictors of coronary risk [1,2], and a exciting therapeutic strategy that uses reconstituted or recombinant (r) HDL has recently been developed. Recently, Parolini et al. reported the effects of rHDL (ETC-216) on rabbit lipid-rich soft plaques [3]. In ETC-216-treated animals, a significant regression of plaque volume was seen at higher doses. In addition, post-treatment magnetic resonance imaging showed smaller plaques in (Apo)A-I(Milano)-treated rabbits compared to the placebo [4]. In humans, Nissen et al. showed that ETC-216 administered intravenously at weekly intervals produced a significant regression of coronary atherosclerosis (−7.2%) as measured by intravascular ultrasound (IVUS) [5]. After the infusion of ETC-216, the regression of coronary atherosclerosis is accompanied by reverse remodeling of the external elastic membrane, resulting in no change in luminal dimensions as assessed by IVUS [6]. These results support the notion that short-term infusions are rapidly effective at preventing the progression of the atherosclerosis burden. Although studies are underway on the use of ApoA-I mimetic peptides (D4F, L37pA, etc.) [7–9], none of them is clinically available for use. Therefore, the newly developed compound ETC-642, a peptide/phospholipid complex in which the peptide (22 amino acids) is much shorter than the 243-amino acid ApoA-I while retaining the amphipathic helical structure of ApoA-I and the ability to associate with lipids, was used in this study.

We recently established a method for the precise measurement of plaque volume as well as plaque area by IVUS in the thoracic descending aorta in the Watanabe heritable hyperlipidemic-myocardial infarction (WHHL-MI) rabbit [10,11]. In addition, we have also devised a strategy to quantitatively analyze the changes

* Corresponding authors. Tel.: +81 928011011; fax: +81 928652692.
E-mail addresses: miuras@cis.fukuoka-u.ac.jp (S. Miura), saku-k@fukuoka-u.ac.jp (K. Saku).

0021-9150/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2011.05.029

in lipoprotein subfractions after rHDL containing ApoA-I and 1-palmitoyl-2-oleoyl-phosphatidyl-choline (POPC) (ApoA-I/POPC discs) infusion by capillary isotachophoresis (cITP) [12]. Therefore, the present study examined the effects of ETC-642 on changes in atherosclerotic plaque area and volume by IVUS and plasma lipoprotein subfractions by cITP in WHHL-MI rabbits.

2. Methods

2.1. Materials

ESP 24218 is a synthetic peptide. As shown in Supplementary Fig. S1, ETC-642 (kindly provided by Pfizer Inc., NY, USA) is a peptide/phospholipid complex composed of ESP 24218, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and sphingomyelin (SM). The mass ratio of SM/DPPC/ESP 24218 is 3.75/3.75/1. Discoidal rHDL containing POPC and full-length ApoA-I (initial ApoA-I/POPC molar ratio 100/1) (ApoA-I/POPC) was kindly provided by Rye et al. [13].

2.2. Experimental protocols

2.2.1. Experiment 1 – in vitro cholesterol efflux

Cholesterol efflux was analyzed using ApoA-I, HDL, ApoA-I/POPC or ETC-642. Blood from normal healthy volunteers was collected. Plasma was immediately separated at 4 °C by centrifugation and collected. HDL ($1.063 < d < 1.21$) was then purified by sequential ultracentrifugation, dialyzed. Human peripheral blood monocytes from normal healthy volunteers were also isolated using Lymphoprep. At near-confluence, macrophages were washed with phosphate buffer and incubated for 20 h at 37 °C with 2.5 μCi [1,2-^{3}H]-cholesterol (51.7 μCi/mmol, Perkin Elmer), which was complexed with bovine serum albumin (BSA). After radiolabeling, cholesterol pools were equilibrated for 18 h by incubation with serum-free in the presence of unlabeled cholesterol (30 μg/ml). To determine cholesterol efflux, the macrophages were washed with ice-cold PBS and incubated for 5 h with 300 μl DMEM containing BSA alone or BSA plus ApoA-I as well as the additives (ApoA-I, HDL, ApoA-I/POPC or ETC-642) indicated in Section 3. After incubation for 4 h, cells and media were harvested separately. Radioactivity was measured by scintillation spectrometry of the supernatant and the cell monolayers after lysis with 500 μl 0.15 mol/l NaCl with 0.2 mol/l NaOH. Fractional cholesterol efflux was calculated as cpmmedium/(cpmmedium + cpmcells) × 100%.

2.2.2. Experiment 2 – in vitro incubation experiment procedures

To analyze the effects of ETC-642 on cITP, HDL and LDL subfractions were examined by incubating whole plasma in the presence of ETC-642 at a peptide concentration of 0.5 mg/ml at 37 °C for 2 h. Incubation was stopped by cooling the samples on ice. cITP of lipoproteins in serum was performed on a Beckman P/ACE MDQ system (Beckman-Coulter, Inc., Tokyo, Japan) according to the method of Böttcher et al. [14] with some modifications [15,16]. For routine analysis, 6 μl of serum was diluted with 14 μl of leading buffer (LB) consisting of 10 mM HCl and 18 mM ammediol (2-amino-2-methyl-1,3-propanediol) (pH 8.8), prestained with 10 μl of 0.1 mg/ml ceramide, which was prepared by dissolving 1 mg of ceramide in 1 ml of anhydrous methanol and diluting 10-fold with anhydrous ethylene glycol for 1 min at room temperature, and mixed with 50 μl of a mixture containing LB with 0.35% hydroxypropylmethylcellulose, spacers, and 5-carboxy-fluorescein as an internal marker.

2.2.3. Experiment 3 – in vivo experiment to examine acute effects

Bolus intravenous drug infusion into Japanese White (JW) or WHHL-MI rabbits was performed using ETC-642 at a peptide concentration of at 50 mg/kg. Blood was drawn before and at 2 h and 24 h after infusion. Lipoprotein subfractions in whole plasma were analyzed by cITP.

2.2.4. Experiment 4 – in vivo experiment to examine chronic effects

Twenty-one WHHL-MI rabbits (F/M: 12/9, age: 7–11 months, BW: 2.6–3.2 kg) fed normal rabbit chow were randomly assigned to be injected intravenously with phosphate buffer (control group, $n = 8$) or ETC-642 at a peptide concentration of 15 mg/kg (low-dose group, $n = 5$) or 50 mg/kg (high-dose group, $n = 8$). The first IVUS was performed after blood was drawn (pre). Intravenous drug injection was started 4–6 days after the first IVUS. Drug was injected into rabbits twice a week for 12 weeks. Blood was draw again at 4, 8, and 12 weeks (post) of drug infusion. Blood biochemical parameters and lipid profile were analyzed at pre and post. Separation of plasma lipoproteins by ultracentrifugation and analysis of lipoprotein subfractions by cITP were also performed.

2.3. Drug infusion

ETC-642 drug for infusion was prepared by hydrating 500 mg lyophilized powder with 50 ml buffer (final concentration, 10 mg/ml). Injection volumes (ml) of ETC-642 solution for the low-dose and high-dose groups were calculated as 1.5 × BW (kg) and 5 × BW (kg), respectively. Drug injection was performed via an auricular vein at 0.5 ml/min using a syringe infusion pump. Each rabbit was infused a total of 24 times during the 3-month period.

2.4. Analysis of plasma lipoprotein subfractions by cITP analysis using whole plasma and separation of plasma lipoproteins by ultracentrifugation

Whole plasma in healthy subjects and JW or WHHL rabbits were used for cITP analysis. In addition, to identify small-dense (sd) LDL and large buoyant (lb) LDL in LDL subfractions in plasma from WHHL rabbits, we used a quantitative ultracentrifugation method to separate plasma lipoproteins for cITP analysis, as described previously [15]. Since apoB-containing lipoproteins are susceptible to modification after being separated from HDL and plasma proteins ($d > 1.063$ g/ml plasma fraction), cITP LDL subfractions were analyzed by cITP in triglyceride-rich lipoprotein-depleted plasma ($d > 1.019$ g/ml plasma fraction). cITP small, sdLDL + HDL subfractions were analyzed in the plasma $d > 1.040$ g/ml fraction. sdLDL subfractions were calculated from sdLDL + HDL subfractions and HDL subfractions measured in plasma $d > 1.040$ and 1.063 fractions. lbLDL were calculated from LDL subfractions measured in plasma $d > 1.019$ and 1.040 fractions. cITP analysis was performed as described previously [15,16].

2.5. Evaluation of atherosclerosis by IVUS

Atherosclerotic plaque in rabbit aorta was evaluated by IVUS as described previously [11]. The first and second IVUS were performed from the left and right femoral arteries, respectively. Three-dimensional images were obtained by manual tracing of the lumen and vessel area (LA and VA); a series of cross-sections of ultrasonographic images was selected exactly 1.0 mm apart along the long axis of the vessel. IVUS analyses included the vessel volume (VV), lumen volume (LV), and plaque volume (PV). Plaque area (PA) or PV was calculated as VA minus LA or VV minus LV, respectively.

2.6. Statistical analysis

All of the data analyses were performed using the Statistical Analysis System Software Package (Ver. 9.1, SAS Institute Inc.,

Cary, NC, USA) at Fukuoka University (Fukuoka, Japan). Categorical variables were compared between groups by a chi-square analysis. Significant changes in continuous variables during the study period were examined by the Wilcoxon sign-rank test or repeated measures analysis of variance. Differences in continuous variables among the three groups were examined by an analysis of variance (ANOVA) and Dunnett's multiple comparison tests. The linear trends in the changes of variables across drug treatment groups were examined by non-parametric ANOVA and Dunnett's multiple comparison tests. The Spearman correlation was used to examine the correlation among continuous variables. Data are presented as mean ± standard error (S.E.), unless indicated otherwise. A p value of less than 0.05 was considered to reflect significance, unless indicated otherwise.

3. Results

3.1. Experiment 1

3.1.1. ETC-642 had a similar effect on cholesterol efflux in macrophage cells as ApoA-I/POPC

Since rHDL is known to be involved in cholesterol efflux from cells, we first examined the effect of ETC-642 in human macrophage cells. As shown in Supplementary Fig. S1, ETC-642 enhanced cholesterol efflux in a dose-dependent manner compared with ApoA-I, and ETC-642 (5–10 μg/ml) had an effect on cholesterol efflux comparable to that of ApoA-I/POPC.

3.2. Experiment 2

3.2.1. Acute effects of ETC-642 incubated with human plasma

In healthy subjects, cITP separates HDL into three subfractions: fast (f)-, intermediate (i)- and slow (s)-migrating HDL (peaks 1, 2 and 3, respectively), plasma triglyceride-rich lipoprotein (TRL) into two subfractions: fast- and slow-migrating TRL (fTRL and sTRL, peaks 4 and 5), and LDL into two major subfractions: fast- and slow-migrating LDL (fLDL and sLDL, peaks 6 and 7) (Fig. 1A). Incubation of whole plasma from healthy subjects with ETC-642 caused a drastic decrease in fHDL and a marked increase in sHDL. Since these results indicate that ETC-642 remodeled fHDL into sHDL (pre-β HDL fraction) in plasma, ETC-642 is a producer of pre-β HDL. Various forms of atherogenic modified LDL, including sdLDL and oxidized-LDL, have an increased negative charge. fLDL represents charge-modified LDL and sLDL represents normal LDL [15]. In addition, we previously reported that ApoA-I/POPC remodeled plasma TRL and LDL into particles with slower electrophoretic mobility after incubation with human plasma and also increased the size of LDL [15,16]. ETC-642 increased peak 7 after 30 min of incubation with human plasma compared to before incubation (Fig. 1A-ab) and remodeled plasma TRL and LDL into particles with slower electrophoretic mobility.

3.3. Experiment 3

3.3.1. Bolus infusion of ETC-642 into Japanese White (JW) and WHHL-MI rabbits on lipid profiles as determined by cITP

Infusion of ETC-642 after 2 h caused a marked increase in sHDL (peak 3) in both JW and WHHL-MI rabbits. ETC-642 increased peak 7 after bolus infusion into JW rabbits compared to before infusion (Fig. 1B-ac). In WHHL-MI rabbits, normal fLDL and sLDL are absent (peaks 6 and 7), and only charge-modified LDL is seen [very-very-fast (vvf) and very-fast (vf) LDL subfractions (peaks 4 and 5)]. Therefore, vvfLDL and vfLDL were the major LDL subfractions in WHHL-MI rabbits. ETC-642 decreased peaks 4 and 5 and increased peaks 6 and 7 after 2 h bolus infusion into WHHL-MI rabbits compared to before infusion (Fig. 1B-bd). In addition, ETC-642 increased peak 7 at 24 h after bolus infusion into WHHL-MI rabbits compared to 2 h after infusion. ETC-642 also remodeled plasma LDL into particles with slower electrophoretic mobility. On the other hand, at 24 h after infusion of ETC-642 there was no increase in sHDL (peak 3) in either JW or WHHL-MI rabbits (Fig. 1B-ce).

3.4. Experiment 4

3.4.1. Changes in body weight (BW) and biological parameters in the control, low-dose and high-dose groups

With regard to the changes in biological parameters in the control, low-dose and high-dose WHHL-MI rabbits, age and BW were similar among the groups (Supplementary Table S1). In addition, there were no adverse effects on liver and renal function even with high-dose ETC-624.

In the high-dose group, the levels of total cholesterol (TC), triglyceride (TG) and HDL cholesterol were significantly increased after 12 weeks of drug infusion, while LDL cholesterol was significantly decreased. On the other hand, there were no changes in the levels of TC, TG, LDL cholesterol or HDL cholesterol after 12 weeks of drug infusion in the control and low-dose groups.

3.4.2. Changes in IVUS parameters before and after 12 weeks of drug infusion in the control, low-dose and high-dose groups

Fig. 2 shows the representative images of IVUS analysis and changes in IVUS parameters before and after 12 weeks of drug infusion in the control, low-dose and high-dose groups. Representative images before and after 12 weeks of drug injection in the control and high-dose groups in WHHL rabbits were shown in Fig. 3A. %PV in the high-dose group did not show significant change before and after infusion (30.9–28.6%), whereas %PV in the control group increased from 27.8% to 37.9%.

There were no significant differences in the changes in VV (Fig. 2B-a). Changes in LV in control rabbits were significantly decreased, and changes in LV in the high-dose group were significantly higher than those in the control group (Fig. 2B-b). Changes in PV and percent (%) PV in control rabbits were significantly increased, and changes in PV and %PV in the high-dose group were significantly lower than those in the control group (Fig. 3B-cd).

3.4.3. Changes in lipid parameters in plasma ultracentrifugation fractions before and after 12 weeks of drug infusion in the control, low-dose and high-dose groups

Fig. 3 shows changes in cITP-measured lipoprotein parameters before and 12 weeks after infusion in the control, low-dose and high-dose groups. ETC-642 significantly changed the distribution of charge-modified LDL subfractions in the high-dose group. Although ETC-642 drastically changed the distribution of the HDL subfraction in a short-term (30 min) in vitro incubation experiment (Experiment 2, Fig. 1A) and in an in vivo experiment in the acute phase (Experiment 3, Fig. 1B), there were no significant changes in HDL subfractions after 12 weeks. Instead, it decreased vvfLDL (peak 4) but increased vfLDL (peak 5) in the low-dose and high-dose groups (Fig. 3B-ab). In addition, low and high doses of ETC-642 increased lbLDL and decreased sdLDL, indicating that ETC-642 converted sdLDL into lbLDL (Fig. 3B-cd). In particular, the high-dose group, but not the low-dose group, showed a significant reduction of vvfLDL and sdLDL and an increase in vfLDL and lbLDL throughout the study period.

3.4.4. Association between IVUS parameters and changes in lipid parameters in plasma and plasma ultracentrifugation fractions

Changes in the levels of HDL cholesterol and LDL-C were positively and negatively correlated with changes in %PV in all of the rabbits (Fig. 4A and B). In addition, changes in %PV in all of

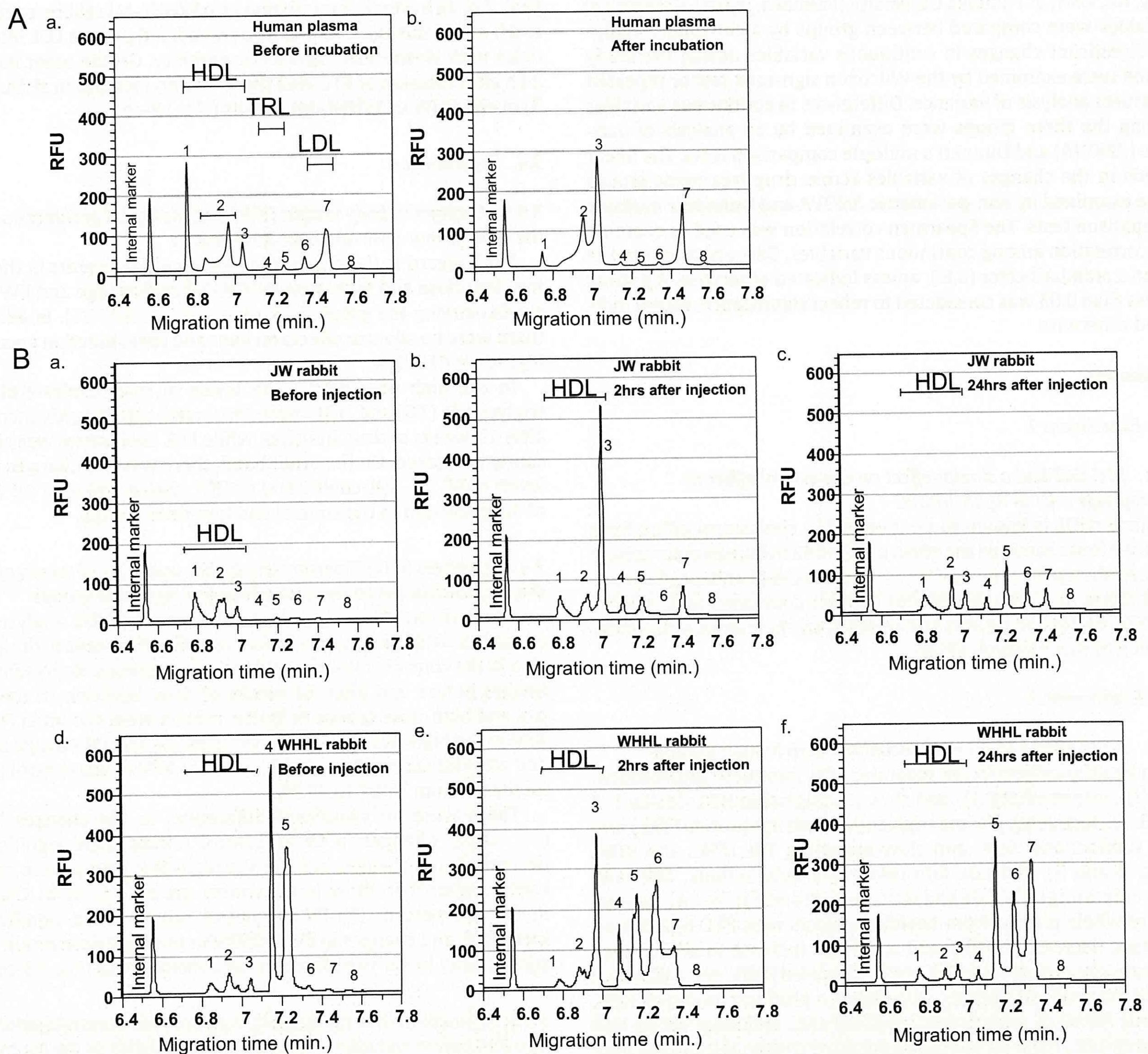


Fig. 1. (A) Acute effects of the incubation of ETC-642 with human plasma for 2 h on lipid profiles as determined by cITP. (B) Acute effects of the bolus injection of ETC-642 before, after 2 h and 24 h into Japanese White (JW) (a–c) and WHHL-MI (c–e) rabbits on lipid profiles as determined by cITP. cITP separates HDL into three subfractions: fast (f)-, intermediate (i)- and slow (s)-migrating HDL (peaks 1, 2 and 3, respectively), plasma TRL into two subfractions: fast- and slow-migrating TRL (fTRL and sTRL, peaks 4 and 5), and LDL into two major subfractions: fast- and slow-migrating LDL (fLDL and sLDL, peaks 6 and 7). Three separate experiments were performed. Lipoprotein was stained by a lipophilic dye and detection was achieved by laser-induced fluorescence at 488 nm. RFU indicates relative fluorescence units.

the rabbits were well-correlated with changes in LDL subfractions. Changes in %PV were positively correlated with changes in vvfLDL and sdLDL, and negatively correlated with changes in lbLDL and sdLDL (Fig. 4C–F). On the other hand, we did not analyze the association between changes in %PV and changes in HDL subfractions because ETC-642 did not change the distribution of the HDL subfraction in the acute phase at 24 h after infusion (Experiment 3).

4. Discussion

The main finding of the present study is that the infusion of high-dose ETC-642 inhibited the progression of atherosclerosis as assessed by IVUS in WHHL rabbits. Moreover, ETC-642 converted more negative-charged modified LDL to less negative-charged LDL as analyzed by cITP, and converted sdLDL into lbLDL after treatment for 12 weeks (Experiment 4). Although ETC-642 drastically changed the distribution of the HDL subfraction in vivo experiment in the acute phase at 2 h after infusion (Experiment 3), there were no significant changes in HDL subfractions (Experiment 4). Since we performed cITP analysis at 48 h after the last infusion in Experiment 4, and since the metabolic turnover rate of pre-β HDL is very fast in rabbits, we cannot observe significant differences in the HDL subfraction over the long term because ETC-642 did not change the distribution of the HDL subfraction in the acute phase at 24 h after infusion (Experiment 3).

There are different kinds of rHDL, including synthetic HDL containing purified ApoA-I, recombinant ApoA-I and ApoA-I mimetic peptides complexed with phospholipids. If ApoA-I mimetic peptides are as effective at enhancing RCT as rHDL containing full-length ApoA-I, they may be useful. Although oral administration of D4F reduced the regression of atherosclerotic plaque in Apo-E-deficient mice [8], no further clinical data have been provided [17]. Another peptide composed of 37 amino acids (L37pA)

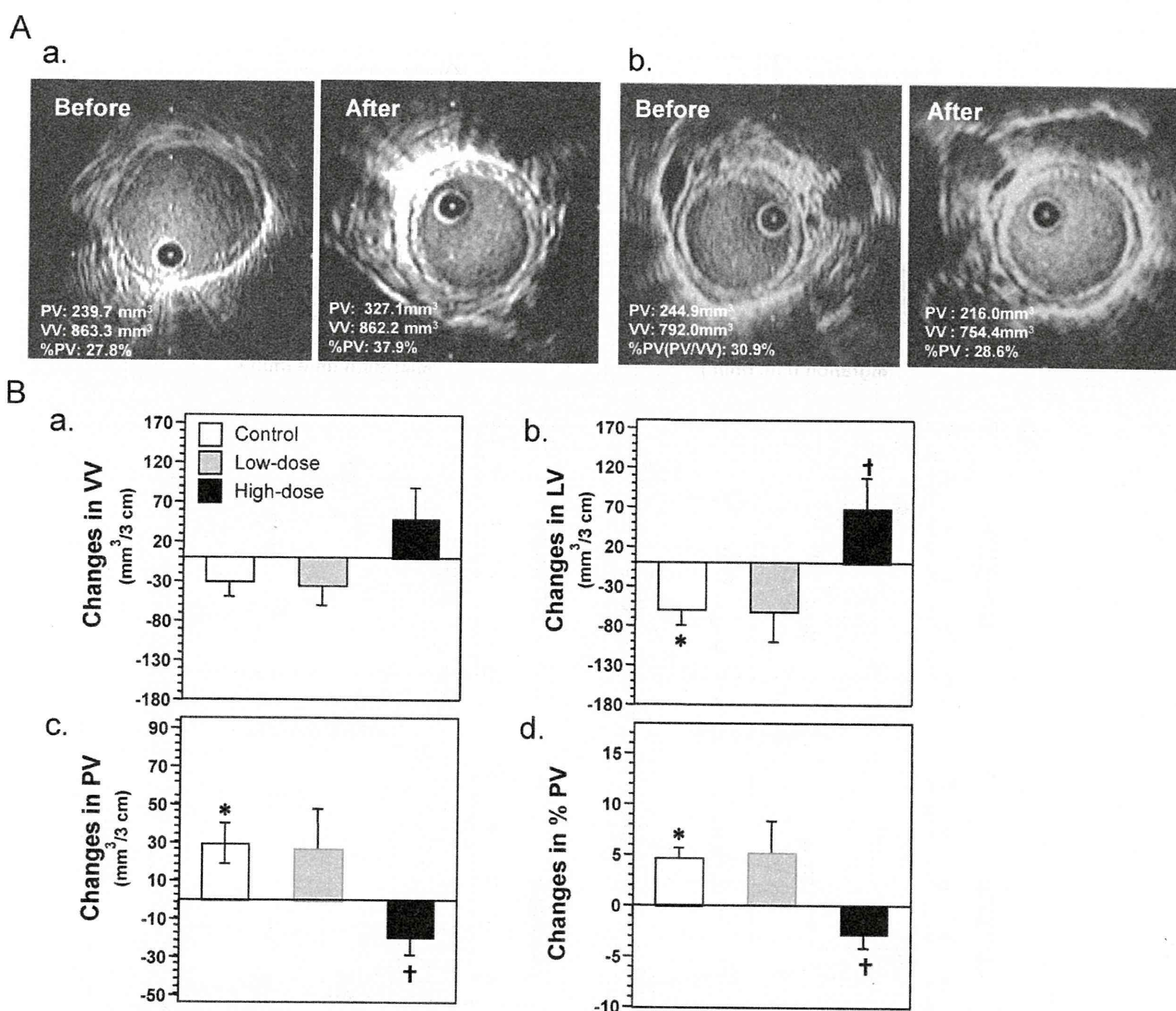


Fig. 2. (A) Representative images of IVUS analysis before and after 12 weeks of drug injection in the control (a) and high-dose (b) groups in WHHL rabbits. (B) Changes in IVUS parameters before and after 12 weeks of drug injection in the control (open bars), low-dose (gray bars) and high-dose (closed bars) groups. Changes in vessel volumes (VV) (a), lumen volumes (LV) (b), plaque volumes (PV) (c) and percent (%) PV (d) were calculated as the values after 12 weeks of drug infusion minus those before drug infusion. *$p < 0.05$ vs. before in each group. †$p < 0.05$ vs. control group.

promotes the efflux of cholesterol and phospholipids from cells [9]. More recently, although ETC-642 found that it was effective in this study, the identify of the best sequence of an ApoA-I mimetic peptide remains unclear.

Generally, the loss of anti-inflammatory function correlated with a loss of function in reverse cholesterol transport. Dysfunctional HDL can be improved by apoA-I mimetic peptides that bind oxidized lipids with high affinity. Although both the increase in RCT and decrease in atherogenic LDL particles by ETC-642 are important mechanisms in the regression of atherosclerosis in this study, the most plausible mechanism of the effect involves the sequestration of oxidized LDL. The infusion of high-dose ETC-642 inhibited the progression of atherosclerosis. According to our data, the ETC-642-induced antiatherogenic effects are mainly due to changes in LDL charge, since ETC-642 decreased vvfLDL (more negatively charged than very-fast LDL) but increased vfLDL, which indicates that ETC-642 converted more negatively charged LDL subfraction into less negatively charged LDL subfraction. ETC-642 also increased lbLDL and decreased sdLDL, indicating that ETC-642 converted sdLDL into lbLDL. In addition, changes in % PV were positively correlated with changes in vvfLDL and sdLDL, and negatively correlated with changes in lbLDL and sdLDL. On the other hand, there were no significant associations between changes in %PV and changes in the HDL subfraction, although changes in the levels of HDL cholesterol were negatively correlated with changes in %PV. Since denatured LDL, such as oxidized-LDL and small-dense LDL, is more negatively charged than native LDL, ETC-642 infusion is potentially anti-atherogenic. In addition, the fact that ETC-642 remodels plasma HDL to increase sHDL in vitro indicates that drastically increased pre-β HDL in HPLC by ETC-642 in patients with stable atherosclerosis [18] could be largely attributable to an interaction between ETC-642 and plasma HDL.

In this study, we also observed that ETC-642 increased TG levels after the infusion of high-dose ETC-642 for 12 weeks in WHHL-MI rabbits, whereas LDL-C levels decreased. Nanjee et al. also reported a significant increase in TG levels after the infusion of ApoA-I/POPC in humans [19]. It is possible that phospholipids in ApoA-I/POPC and ETC-642 increase the secretion of TRL particles. In fact, previous studies have shown that the inhibition of POPC synthesis decreased the secretion of VLDL from cultured rat hepatocytes [20] and that the inhibition of sphingomyelin synthesis decreased plasma TG levels in apoE-knockout mice [21]. It is also possible that

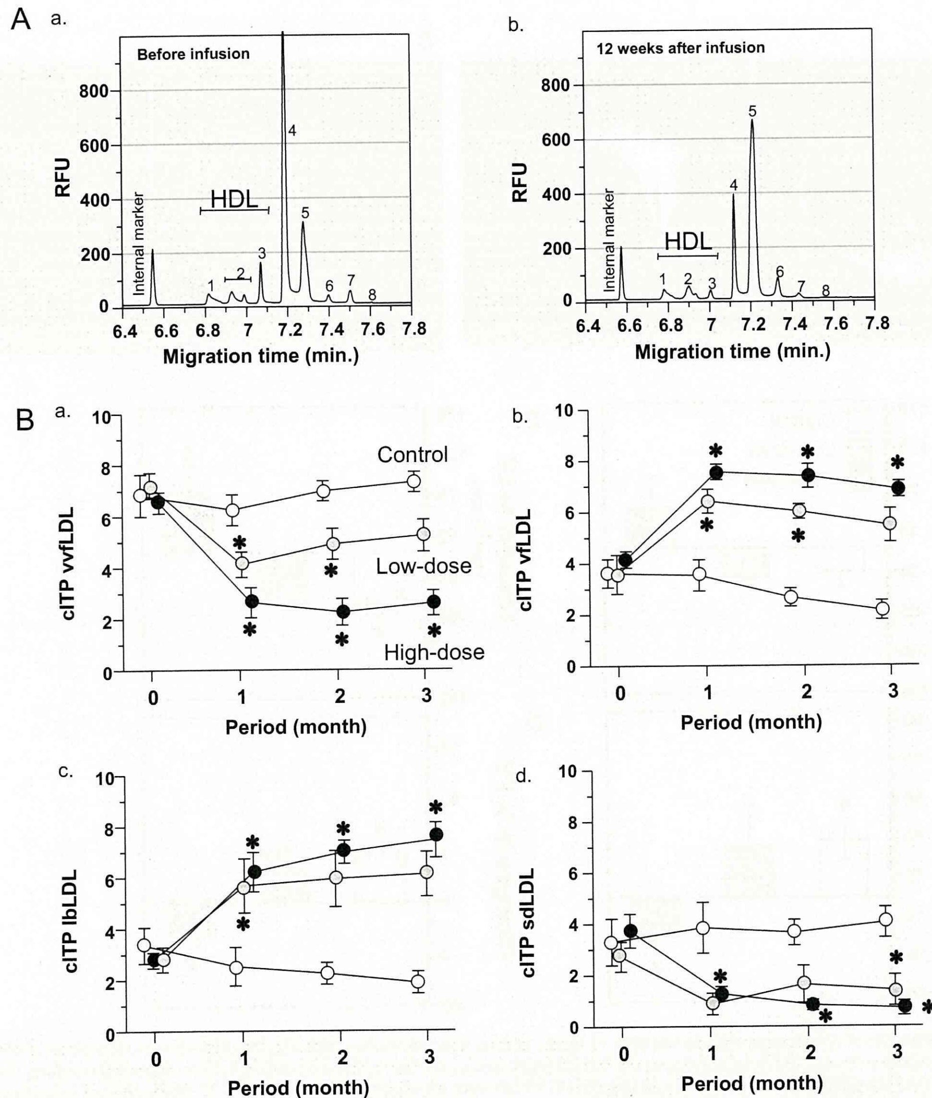


Fig. 3. (A) Representative pictures of changes in lipid parameters in plasma ultracentrifugation fractions before and after 12 weeks of drug infusion in WHHL rabbit with high-dose ETC-642. (B) Time-course of changes in lipid parameters [very-very-fast (vvf) LDL (a), very-fast (vf) LDL (b), large-buoyant (lb) LDL (c) and small-dense (sd) LDL (d)] in plasma ultracentrifugation fractions before and after 4, 8 and 12 weeks of drug injection in WHHL rabbits in the control (open circles), low-dose (gray circles) and high-dose (closed circles) groups. *$p < 0.05$ vs. before in each group. Lipoprotein was stained by a lipophilic dye and detection was achieved by laser-induced fluorescence at 488 nm. RFU indicates relative fluorescence units.

the catabolism of TRL remnants may be delayed by ETC-642 due to interaction with proteins of the lipoprotein transport system.

Although HDL levels in plasma are low in WHHL rabbits [22], we confirmed the function of ETC-642 in plasma by cITP analysis. Recent reports have indicated that the effects of ApoA-I mimetic peptides are mostly due to anti-inflammation [23]. Oxidized lipids in LDL induce monocyte chemotactic protein-1 (MCP-1) expression in endothelial cells (ECs), resulting in monocyte chemotaxis. Although there were no significant differences in inflammation markers (plasma levels of MCP-1 and platelet-activating factor acetylhydrolase) between the high-dose ETC-642, low-dose ETC-642, and control groups in this study (data not shown), we did not analyze the precise role of ETC-642 in ECs, and further investigation is needed.

The preclinical demonstration of RCT and plaque regression is problematic for assessing the effects of drugs. A large number of animals are required to overcome the degree of intra-animal variation inherent in these types of studies. To resolve these problems, we used two important techniques, IVUS and cITP, in an animal model, WHHL-MI rabbits. We performed a longitudinal study, where each animal serves as its own control, which results in less endpoint variability using IVUS. IVUS has emerged as the preferred imaging modality for evaluating the impact of pharmacological therapies on arterial plaque progression and/or regression [11,24]. In addition, we analyzed the thoracic descending aorta, but not carotid artery, which has been analyzed in previous studies [3,24], by IVUS in rabbits. Therefore, we planned to examine the regression or the prevention of progression of plaque in WHHL-MI rabbits which spontaneously develop significant atherosclerotic plaque and are of sufficient size to accommodate serial IVUS imaging. Finally, compared to mouse models, WHHL rabbits resemble humans with regard to lipoprotein metabolism [22]. To evaluate the effects of drugs on lipid profiles, we can generally only measure plasma levels of TG, LDL- and HDL-cholesterol. cITP can separate subfractions

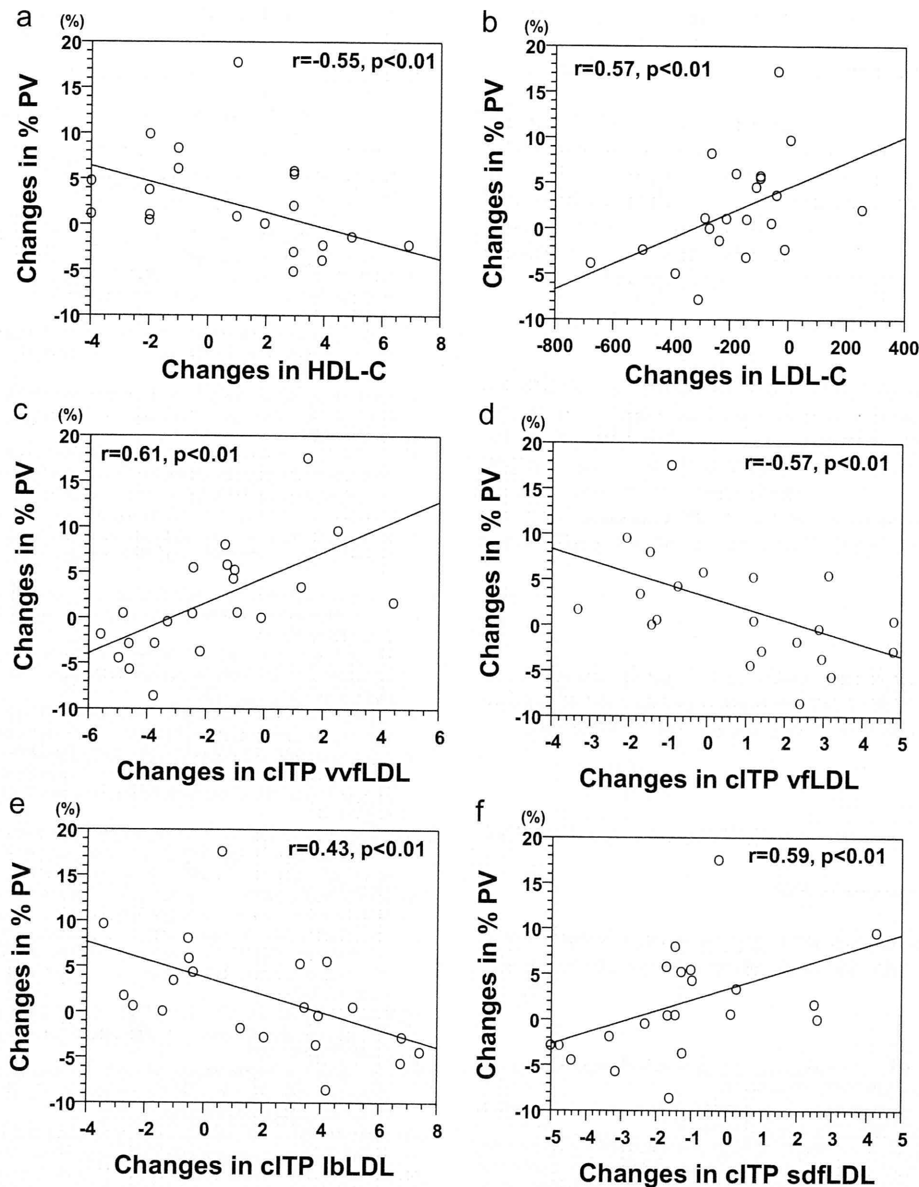


Fig. 4. Association between changes in percent (%) plaque volume (PV) and changes in lipid parameters in the levels of HDL-C (A) and LDL-C (B) and in plasma ultracentrifugation cITP fractions [very-very-fast (vvf) LDL (C), very-fast (vf) LDL (D), large-buoyant (lb) LDL (E) and small-dense (sd) LDL (F)] after 12 weeks of ETC-642 injection.

of HDL and LDL in plasma lipoprotein in detailed lipid profiles and can be used to examine the direct effects of drugs in plasma. In this study, we finally found associations between changes in plaque volume and changes in vvfLDL, sdLDL, vfLDL and lbLDL, and how to use ETC-642 to prevent the progression of plaque in plasma. Thus, these two techniques gave us variable information on the effects of ETC-642.

The present study was designed to use two different doses of ETC642 (15 and 50 mg/kg) because Nissen et al. reported that ETC-216 induced the significant regression of coronary atherosclerosis by IVUS in humans and the two doses used (15 and 45 mg/kg) induced about the same level of regression [5]. Parolini et al. reported that 40 and 150 mg/kg of ETC-216 each induced a significant reduction of plaque volume [3]. In this study, high-dose ETC-642 (50 mg/kg) was more effective than low-dose (15 mg/kg) for preventing the progression of plaque. While ETC-216 produced a regression of plaque volume, ETC-642 inhibited the progression of plaque volume and did not induce regression. There are some important issues regarding these observations. First, although the abdominal aorta was assessed using MRI [4], IVUS was used to analyze the carotid artery [3] but not the thoracic descending aorta. Second, they used New Zealand White rabbits instead of WHHL-MI rabbits. Since WHHL-MI rabbits develop severe atherosclerosis due to hypercholesterolemia even when fed normal chow [9], it may be difficult to induce the regression of plaque by rHDL compared to White rabbits. In fact, experimental atherosclerosis in cholesterol-fed rabbits differs markedly from human plaques. The atherosclerosis in rabbits, induced over several months, contains nearly 90% cholesterol-enriched foam cells [25] that are more easily lipid-depleted [24]. Although lipids represent only 13–16% of average human plaque [26], atherosclerotic plaque formation in the WHHL rabbit is quite similar to that observed in humans [27].

Third, they reported that a higher concentration of ETC-216 (40 and 150 mg/kg) induced a significant reduction of plaque volume. Therefore, we need to define the optimal dose of ETC-642 in further analysis.

There are several study limitations. First, our study lacks a positive-control group; i.e., a group that receives either ETC-216 or only a phospholipid complex. We could not compare ETC-216 with ETC-642, and a phospholipid complex did not enhance cholesterol efflux in an in vitro study because it is very toxic toward the cells. Further study will be needed to confirm the efficacy of ETC-642 using conventional rHDL or ApoA-I mimetic peptides.

5. Conclusions

The ETC-642-induced remodeling of sdLDL to lbLDL may prevent progression of the aortic plaque burden. HDL-based therapy may be useful for preventing the progression of PV. Although the value of the extensive lowering of LDL cholesterol levels by statins in preventing cardiovascular (CV) events has been well documented, the combination of the extensive lowering of LDL cholesterol with the aggressive improvement of HDL function may dramatically prevent CV events.

Sources of funding

This work was supported in part by a Grant-in-Aid for Scientific Research (21590960) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Drs. Saku and Miura).

Disclosure

This work was supported in part by Pfizer Inc., USA (Dr. Saku).

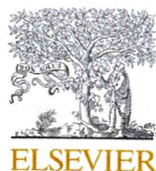
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.05.029.

References

[1] von Eckardstein A, Nofer JR, Assmann G. Acceleration of reverse cholesterol transport. Curr Opin Cardiol 2000;15:348–54.
[2] Gordon DJ, Rifkind BM. High-density lipoprotein – the clinical implications of recent studies. N Engl J Med 1989;321:1311–36.
[3] Parolini C, Marchesi M, Lorenzon P, et al. Dose-related effects of repeated ETC-216 (recombinant apolipoprotein A-I Milano/1-palmitoyl-2-oleoyl phosphatidylcholine complexes) administrations on rabbit lipid-rich soft plaques: in vivo assessment by intravascular ultrasound and magnetic resonance imaging. J Am Coll Cardiol 2008;51:1098–103.
[4] Ibanez B, Vilahur G, Cimmino G, et al. Rapid change in plaque size, composition, and molecular footprint after recombinant apolipoprotein A-I Milano (ETC-216) administration: magnetic resonance imaging study in an experimental model of atherosclerosis. J Am Coll Cardiol 2008;51:1104–9.
[5] Nissen SE, Tsunoda T, Tuzcu EM, et al. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. J Am Med Assoc 2003;290:2292–300.
[6] Nicholls SJ, Tuzcu EM, Sipahi I, et al. Relationship between atheroma regression and change in lumen size after infusion of apolipoprotein A-I Milano. J Am Coll Cardiol 2006;47:992–7.
[7] Navab M, Anantharamaiah GM, Hama S, et al. Oral administration of an ApoA-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. Circulation 2002;105:290–2.
[8] Li X, Chyu KY, Faria Neto JR, et al. Differential effects of apolipoprotein A-I-mimetic peptide on evolving and established atherosclerosis in apolipoprotein E-null mice. Circulation 2004;110:1701–5.
[9] Remaley AT, Thomas F, Stonik JA, et al. Synthetic amphipathic helical peptides promote lipid efflux from cells by an ABCA1-dependent and an ABCA1-independent pathway. J Lipid Res 2003;44:828–36.
[10] Shiomi M, Ito T, Yamada S, Kawashima S, Fan J. Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). Arterioscler Thromb Vasc Biol 2003;23:1239–44.
[11] Iwata A, Miura S, Imaizumi S, Zhang B, Saku K. Measurement of atherosclerotic plaque volume in hyperlipidemic rabbit aorta by intravascular ultrasound. J Cardiol 2007;50:229–34.
[12] Imaizumi S, Miura S, Nakamura K, et al. Antiarrhythmogenic effect of reconstituted high-density lipoprotein against ischemia/reperfusion in rats. J Am Coll Cardiol 2008;51:1604–12.
[13] Rye KA, Garrety KH, Barter PJ. Changes in the size of reconstituted high density lipoproteins during incubation with cholesteryl ester transfer protein: the role of apolipoproteins. J Lipid Res 1992;33:215–24.
[14] Böttcher A, Schlosser J, Kronenberg F, et al. Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions. J Lipid Res 2000;41:905–15.
[15] Zhang B, Uehara Y, Hida S, et al. Effects of reconstituted HDL on charge-based LDL subfractions as characterized by capillary isotachophoresis. J Lipid Res 2007;48:1175–89.
[16] Zhang B, Kaneshi T, Ohta T, Saku K. Relation between insulin resistance and fast-migrating LDL subfraction as characterized by capillary isotachophoresis. J Lipid Res 2005;46:2265–77.
[17] Chiesa G, Parolini C, Sirtori CR. Acute effects of high-density lipoproteins: biochemical basis and clinical findings. Curr Opin Cardiol 2008;23:379–85.
[18] Khan M, Lalwani ND, Drake SL, Crokatt JG, Dasseux JH. Single-dose intravenous infusion of ETC-642, a 22-mer apoA-I analogue and phospholipid complex, elevates HDL-C in atherosclerosis patients. Circulation 2003;108(Suppl. IV):563–4.
[19] Nanjee MN, Doran JE, Lerch PG, Miller NE. Acute effects of intravenous infusion of ApoA1/phosphatidylcholine discs on plasma lipoproteins in humans. Arterioscler Thromb Vasc Biol 1999;19:979–89.
[20] Nishimaki-Mogami T, Suzuki K, Takahashi A. The role of phosphatidylethanolamine methylation in the secretion of very low density lipoproteins by cultured rat hepatocytes: rapid inhibition of phosphatidylethanolamine methylation by bezafibrate increases the density of apolipoprotein B48-containing lipoproteins. Biochim Biophys Acta 1996;1304:21–31.
[21] Park TS, Panek RL, Mueller SB, et al. Inhibition of sphingomyelin synthesis reduces atherogenesis in apolipoprotein E-knockout mice. Circulation 2004;110:3465–71.
[22] Shiomi M, Ito T. The Watanabe heritable hyperlipidemic (WHHL) rabbit, its characteristics and history of development: a tribute to the late Dr Yoshio Watanabe. Atherosclerosis 2009;207:1–7.
[23] Shah PK, Chyu KY. Apolipoprotein A-I mimetic peptides: potential role in atherosclerosis management. Trends Cardiovasc Med 2005;15:291–6.
[24] Chiesa G, Monteggia E, Marchesi M, et al. Recombinant apolipoprotein A-I(Milano) infusion into rabbit carotid artery rapidly removes lipid from fatty streaks. Circ Res 2002;90:974–80.
[25] Chiesa G, Di Mario C, Colombo N, et al. Development of a lipid-rich, soft plaque in rabbits, monitored by histology and intravascular ultrasound. Atherosclerosis 2001;156:277–87.
[26] Kragel AH, Reddy SG, Wittes JT, Roberts WC. Morphometric analysis of the composition of coronary arterial plaques in isolated unstable angina pectoris with pain at rest. Am J Cardiol 1990;66:562–7.
[27] Watanabe Y. Serial inbreeding of rabbits with hereditary hyperlipidemia (WHHL-rabbit). Atherosclerosis 1980;36:261–8.

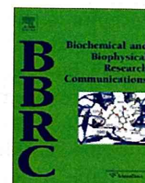
Biochemical and Biophysical Research Communications 407 (2011) 656–662



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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



Species differences of macrophage very low-density-lipoprotein (VLDL) receptor protein expression

Sadao Takahashi [a,b,*], Takashi Ito [c], Yasuo Zenimaru [a], Jinya Suzuki [a], Isamu Miyamori [a], Masao Takahashi [d], Masafumi Takahashi [e], Takafumi Ishida [f], Tatsuro Ishida [g], Ken-ichi Hirata [g], Tokuo T. Yamamoto [h], Tadao Iwasaki [i], Hiroaki Hattori [i], Masashi Shiomi [c,j]

[a] Third Department of Internal Medicine, University of Fukui, Faculty of Medical Science, Japan
[b] Research and Education Program for Life Science, University of Fukui, Faculty of Medical Science, Japan
[c] Institute for Experimental Animals, Kobe University Graduate School of Medicine, Japan
[d] Department of Cardiovascular Surgery, Hiratsuka Kyosai Hospital, Japan
[e] Division of Bioimaging Sciences, Center for Molecular Medicine, Jichi Medical University, Japan
[f] Department of Cardiovascular Medicine, Hiroshima University Graduate School of Biomedical Sciences, Japan
[g] Division of Cardiovascular Medicine, Kobe University Graduate School of Medicine, Japan
[h] Center for Advanced Genome Research, Institute of Development, Aging, and Cancer, Tohoku University, Japan
[i] Department of Advanced Medical Technology and Development, BML, Inc., Japan
[j] Section of Animal Models for Cardiovascular Diseases, Kobe University Graduate School of Medicine, Japan

ARTICLE INFO

Article history:
Received 13 March 2011
Available online 21 March 2011

Keywords:
Atherosclerosis
Foam cell formation
Macrophages
Species differences
VLDL receptor
LDL receptor

ABSTRACT

Triglyceride-rich lipoproteins (TGRLs) and low-density-lipoprotein (LDL) cholesterol are independent risk factors for coronary artery disease. We have previously proposed that the very low-density-lipoprotein (VLDL) receptor is one of the receptors required for foam cell formation by TGRLs in human macrophages. However, the VLDL receptor proteins have not been detected in atherosclerotic lesions of several animal models. Here we showed no VLDL receptor protein was detected in mouse macrophage cell lines (Raw264.7 and J774.2) or in mouse peritoneal macrophages in vitro. Furthermore, no VLDL receptor protein was detected in macrophages in atherosclerotic lesions of chow-fed apolipoprotein E-deficient or cholesterol-fed LDL receptor-deficient mice in vivo. In contrast, macrophage VLDL receptor protein was clearly detected in human macrophages in vitro and in atherosclerotic lesions in myocardial infarction-prone Watanabe-heritable hyperlipidemic (WHHLMI) rabbits in vivo. There are species differences in the localization of VLDL receptor protein in vitro and in vivo. Since VLDL receptor is expressed on macrophages in atheromatous plaques of both rabbit and human but not in mouse models, the mechanisms of atherogenesis and/or growth of atherosclerotic lesions in mouse models may be partly different from those of humans and rabbits.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

The role of low-density-lipoprotein cholesterol (LDL-C) in the development and progression of atherosclerosis has been established in humans [1]. Both qualitative and quantitative abnormalities in circulating triglyceride-rich lipoproteins (TGRLs) may be key factors in the development of human coronary artery diseases (CADs) [2]. The development of macrophage foam cells that contain massive amounts of cholesterol ester is a hallmark of both early and advanced atherosclerotic lesions [3]. There are two major lipoproteins that contribute to macrophage foam cell formation. One is LDL and the other is VLDL. LDL particles that infiltrate into arterial subendothelial regions are oxidized and oxidized LDL (oxLDL) particles are taken up by macrophages through scavenger receptors. VLDL particles without oxidative modification are taken by macrophages through VLDL receptors and remnant receptors in human macrophage cell lines (phorbol-12-myristate-13-acetate: PMA-treated THP-1 monocytic leukemia cells and HL-60 cells) and in human monocyte-derived macrophages [4,5]. In mouse peritoneal macrophages, however, LDL-receptor-related protein-1 (LRP-1) and the LDL receptor that recognizes TGRLs are candidate receptors for mediation of macrophage foam cell formation [6,7].

We were the first to clone and characterize VLDL receptor cDNAs from rabbit heart and human THP-1 cells [8,9]. The VLDL

Abbreviations: TGRLs, triglyceride-rich lipoproteins; VLDL, very low-density lipoprotein; LDL, low-density-lipoprotein; WHHLMI, myocardial infarction-prone Watanabe-heritable hyperlipidemic; apo, apolipoprotein; CADs, coronary artery diseases.

* Corresponding author at: Third Department of Internal Medicine, University of Fukui, Faculty of Medical Science, Japan. Fax: +81 776 61 8111.
E-mail address: sadaost@u-fukui.ac.jp (S. Takahashi).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.03.069

receptor is abundantly expressed in tissues that are active in fatty acid metabolism (heart, skeletal muscle and fat) as well as in brain and macrophages. The ligand specificity of the VLDL receptor is different from that of the LDL receptor. The VLDL receptor only binds to apolipoprotein (apo) E-containing particles such as VLDL and intermediate-density lipoprotein (IDL) obtained from Watanabe-heritable hyperlipidemic (WHHL) rabbits as well as to β-VLDL obtained from cholesterol-fed rabbits. These findings indicated that the VLDL receptor is a lipoprotein receptor for TGRLs, but not for LDL. ApoE and LPL, which are secreted by heart, skeletal muscle, fat and macrophages, accelerate the binding of TGRLs to the VLDL receptor [10]. We proposed that the VLDL receptor functions as a peripheral lipoprotein receptor in tissues active in fatty acid metabolism even though it is true that the VLDL receptor and apoE receptor 2 (ApoER2) are reelin receptors in brain [11,12].

Previous studies have demonstrated that the VLDL receptor protein and mRNA are detected in human atherosclerotic lesions [13,14]. Although the expression of VLDL receptor mRNA in atherosclerotic lesions of rabbits was observed [15,16], no studies have shown VLDL receptor protein in atherosclerotic lesions of rabbits and mice. Detection of VLDL receptor protein in atherosclerotic lesions is important in understanding the mechanisms of atherogenesis and growth of atherosclerotic lesions. We were fortunate enough to obtain a rabbit polyclonal antibody that reacts with human, rabbit, rat and mouse heart VLDL receptor proteins. Using this antibody, we found definite species differences in macrophage VLDL receptor protein expression.

2. Materials and methods

2.1. Reagents

Phorbol-12-myristate-13-acetate (PMA) was purchased from Wako Pure Chemical Industries (Osaka, Japan). RPMI-1640, DMEM

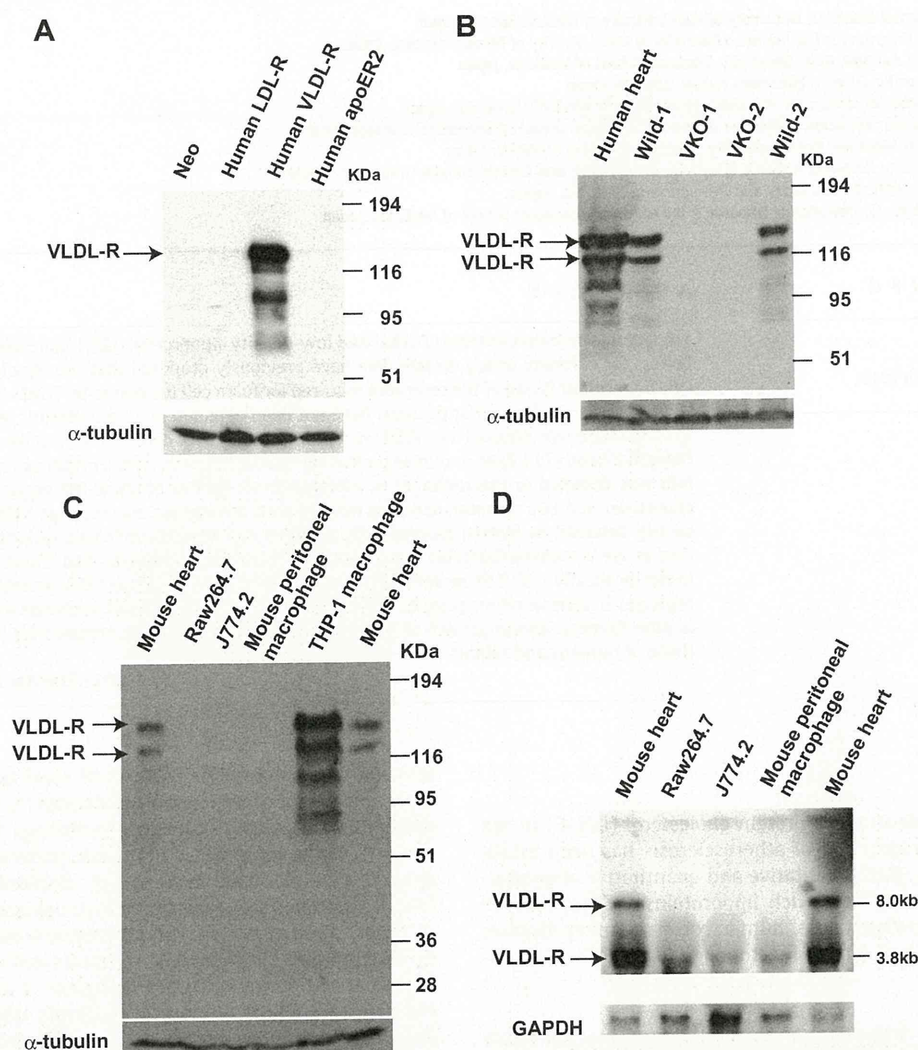


Fig. 1. Characterization of the rabbit polyclonal antibody (VR2) and in vitro assay of VLDL receptor expression in mice and humans. (A) The pSV2-neo plasmid encoding the full-length human LDL receptor (LDL-R), human VLDL receptor (VLDL-R), human apoE receptor 2 (apoER2) cDNA or control pSV2-neo alone (Neo) was transfected into a mutant Chinese hamster ovary cell line lacking LDL receptors (ldlA-7 cells) and G418 selection was performed. Cell lysates were analyzed by Western blot using VR2. (B) Heart tissues from human after Batista operation, two wild-type (Wild-1/-2) and two VLDL-receptor knockout (VKO-1/-2) mice were lysed and analyzed by Western blot using VR2. The higher molecular band is the type 1 VLDL receptor protein and lower band is the mixture of proteins of type 1 VLDL receptor precursor and type 2 VLDL receptor. For Fig. 1A and B, α-tubulin was blotted as a loading control. (C) Cell lysates of the mouse macrophage cell lines (Raw264.7 and J774.2), mouse peritoneal macrophages and PMA-induced human THP-1 macrophage cells were analyzed by Western blot using the VR2 antibody. Mouse heart from wild-type mice was used as a positive control for VLDL receptor proteins. (D) VLDL receptor and GAPDH mRNA expression in the indicated cells and tissues was analyzed by Northern blot analysis.

and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). All other chemicals used were of the highest reagent grade.

2.2. Animals

For detection of heart VLDL receptor protein using antibody, we used C57BL/6J mice from Charles River Laboratories International, Inc. (Yokohama, Japan), and VLDL receptor knockout (KO) mice with a C57BL/6J background [17]. For detection of VLDL receptors in atherosclerotic lesions in vivo, we used normal chow-fed myocardial infarction-prone Watanabe-heritable hyperlipidemic (WHHLMI) rabbits (6 months old, male) [18], normal chow-fed apoE KO mice with a C57BL/6 J background [19] (6 months old, male, Jackson Laboratory, Bar Harbor, ME, USA) and 1.25% cholesterol-fed for 12 weeks LDL receptor KO mice with a C57BL/6J background [20] (16 months old, male). The LDL receptor KO mice and VLDL receptor KO mice were kindly provided by Drs. Goldstein J.L. and Brown M.S. (University of Texas, Dallas, TX, USA). All animal procedures were approved by the Animal Care and Use Committee of the University of Fukui or Kobe University Graduate School of Medicine in accordance with NIH Guidelines.

2.3. Human heart tissue

Human heart tissue was obtained from a Japanese patient (47 years old, male) who suffered from severe cardiomyopathy. He kindly allowed us to use his heart sample after Batista operation and we obtained his written informed consent.

2.4. Cell culture

Human THP-1 monocytic leukemia cells and mouse macrophage cell lines (Raw264.7, J774.2) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 (THP-1 and Raw264.7 cells) or DMEM (J774.2 cells) supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37 °C. Antibody specificity was confirmed by Western blot of a membrane fraction of ldlA-7 cells (LDL receptor-deficient CHO cells, provided by Dr. Monty K., Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA) transfected with human type 1 VLDL receptor, human LDL receptor and human ApoER2 cDNA [9,21,22].

2.5. Isolation and culture of mouse peritoneal macrophages

Macrophages were obtained by collection of the peritoneal exudates of male C57BL/6 mice (20–25 g) at 4 days after they had received an intraperitoneal injection (2 ml) of 4% thioglycolate medium. The peritoneal cells were harvested in phosphate-buffered saline. The cells were collected by centrifugation (700g for 5 min at 4 °C). The cells were washed with DMEM, plated on 60-mm tissue culture dishes (Corning, NY, USA) in DMEM containing 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin, and were incubated in 5% CO_2 at 37 °C. After incubation for 1 h, non-adherent cells were removed by replacing the medium with fresh medium. Adherent macrophages were then collected [23].

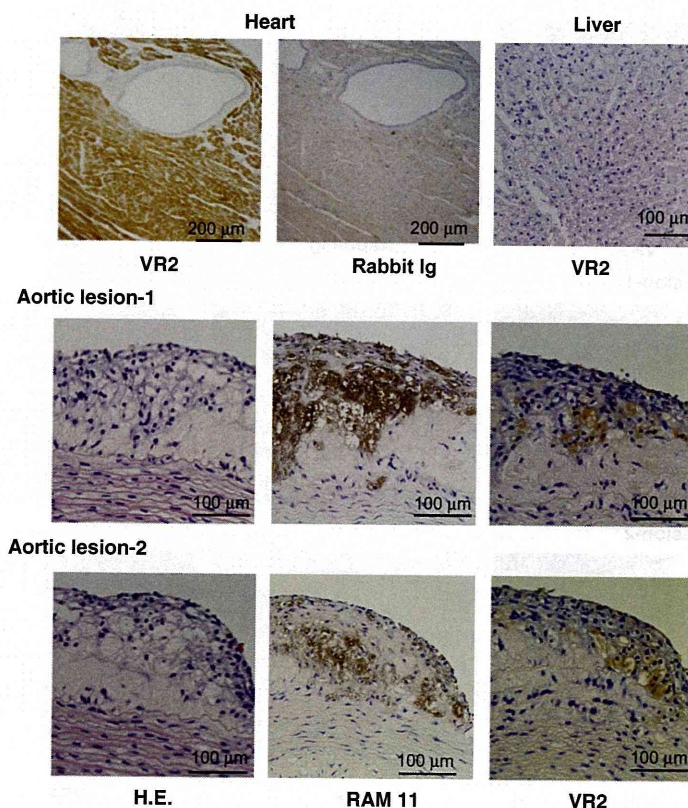


Fig. 2. Immunohistochemical analysis of VLDL receptor protein in WHHLMI rabbits. WHHLMI aorta in 6-month-old male rabbits was immunohistochemically stained for VLDL receptor protein expression using the VR2 antibody (Aortic lesion-1 and -2). Heart and liver tissues were similarly analyzed as VLDL receptor protein-positive and -negative controls, respectively (upper panels). Section of the aorta was also stained with hematoxylin-eosin (HE) or RAM11 (a mouse monoclonal antibody against rabbit macrophages) that was used as a rabbit macrophage marker. For heart sample, rabbit immunoglobulin (Ig) was used as a negative control.

2.6. Anti-VLDL receptor rabbit polyclonal antibody

A synthetic peptide, CASVGHTYPAISVVSTDDDL, which corresponds to the carboxy-terminus of the human, rabbit, rat and mouse VLDL receptors, was synthesized and injected into two Japanese White rabbits and two rabbit polyclonal antibodies from each rabbit was obtained (namely VR1 and VR2) [5]. We showed that VR2 antibody recognized human macrophage VLDL receptor protein in macrophage cell lines (PMA-treated THP-1 and HL-60 cells) and in monocyte-derived macrophages [5]. Then, we confirmed that the VR1 and VR2 antibodies could recognize human, rabbit, rat and mouse heart VLDL receptor proteins. Because VR2 reacted the VLDL receptor protein with higher affinity than VR1, we used VR2 antibody in this study mainly but all results were reconfirmed by VR1.

2.7. Protein isolation and Western blot analysis

Standard methods for Western blotting were used as previously described [24]. Total cell extracts of various types of macrophages (70 μg protein/lane) were analyzed on 7.0% slab SDS–PAGE gels that contained 0.1% SDS. Proteins on the gel were transferred to Immobilon-P membranes (Merck KGaA) using a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). Detection of each first antibody was performed using a proper second antibody and was visualized by enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK).

2.8. Northern blot analysis

A standard method for Northern blotting was used as previously described [10]. Total cellular RNA was isolated using the TRIzol reagent (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA). Total RNA (20 μg per sample) was electrophoresed through a denaturing formaldehyde-agarose gel and was transferred to a Zeta-probe (Bio-Rad Laboratories, Hercules, CA, USA) filter via capillary transfer. After UV cross-linking, the filter was pre-hybridized and then hybridized with mouse VLDL receptor cDNA fragments that were labeled with [α-^{32}P] dCTP (GE) according to the random primer method using the Random Primer DNA Labeling kit Ver. 2 (Takara Bio, Inc., Otsu, Japan).

2.9. Immunohistochemistry

Thoracic aorta, heart and liver tissues from normal chow-fed WHHLMI rabbits (6 months old, male, $n = 3$), normal chow-fed apoE KO mice (6 months old, male, $n = 3$) and LDL receptor KO mice, which were fed 1.25% cholesterol for 12 weeks (16 months old, male, $n = 3$), were examined. Immunohistochemistry was performed as described previously [25]. A rabbit polyclonal antibody against the VLDL receptor (VR2) and a mouse monoclonal antibody (RAM11: Dako, Cambridge, UK) against rabbit macrophages, or a rat monoclonal antibody (BM8: Business Support Center for Biomedical Research Activities, Kobe, Japan) against mouse macrophages (F4/80), were used. Anti-rabbit Envision (Dako, Cambridge,

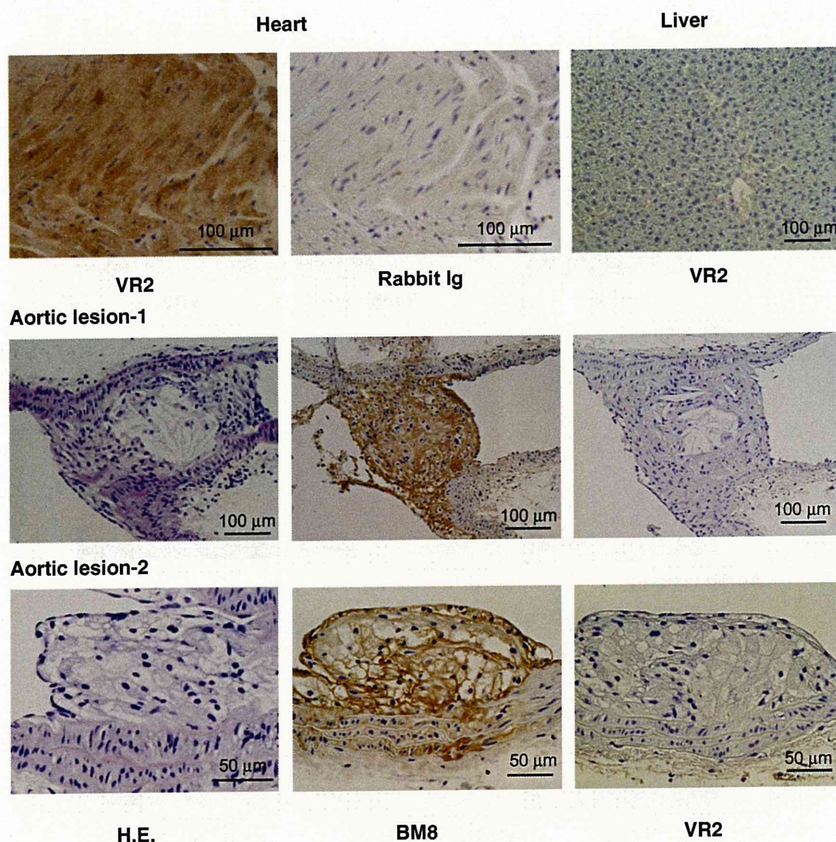


Fig. 3. Immunohistochemical analysis of VLDL receptor protein in atherosclerotic lesions in apoE KO mice. Two different lesions from the aorta of a 6-month-old, male, chow-fed apoE KO mouse are indicated (Aortic lesion-1 and -2). Heart and liver tissues were used as positive and negative controls of VLDL receptor protein, respectively (upper panels). Section of the aorta was also stained with hematoxylin-eosin (HE) or BM8 (a rat monoclonal antibody against mouse macrophages). For heart sample, rabbit immunoglobulin (Ig) was used as a negative control.

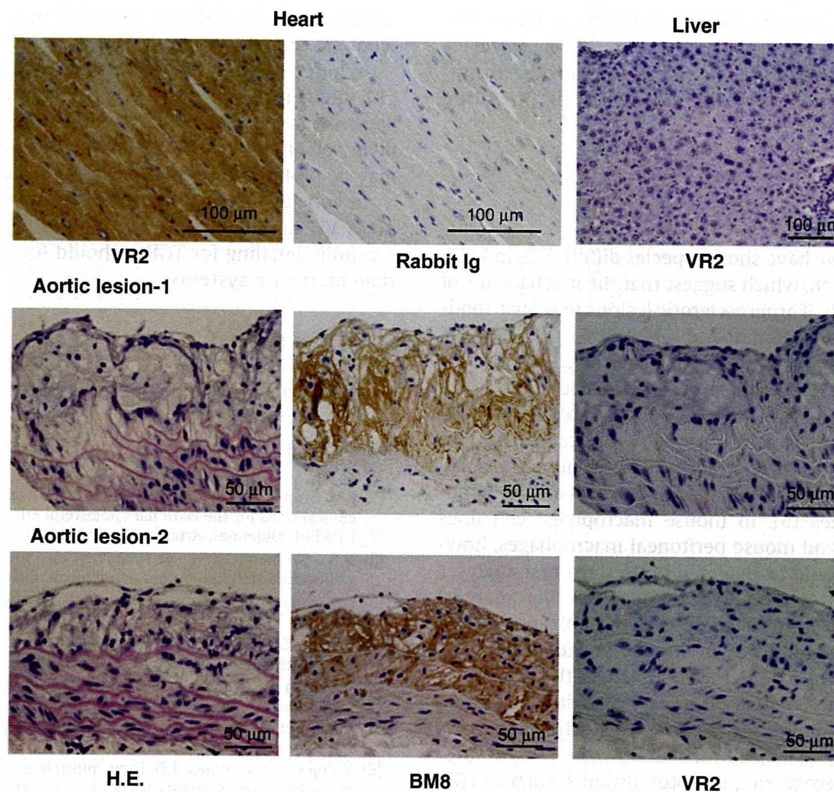


Fig. 4. Immunohistochemical analysis of VLDL receptor protein in atherosclerotic lesions in LDL receptor KO mice. Aorta tissue from a 16-month-old male mouse that was fed a diet of 1.25% cholesterol for 12 weeks is indicated (Aortic lesion-1 and -2). Heart and liver tissues were used as positive and negative controls of VLDL receptor protein, respectively (upper panels). Section of the aorta was also stained with hematoxylin-eosin (HE) or BM8 (a rat monoclonal antibody against mouse macrophages). For heart sample, rabbit immunoglobulin (Ig) was used as a negative control.

UK) or peroxidase-conjugated goat-anti-rat IgG (Cappel, Malvern, PA, USA) was used as a second antibody for VR2, and RAM11 or BM8, respectively.

2.10. Others

Each experimental datum shown in the figures is representative of at least two independent experiments.

3. Results

3.1. Specificity of the anti-VLDL receptor rabbit polyclonal antibody (VR2)

Fig. 1 shows the specificity of the anti-VLDL receptor rabbit polyclonal antibody (VR2). VR2 reacted with only human VLDL receptor-, but not with human LDL receptor- or human ApoER2-cDNA transfected ldlA-7 cells (Fig. 1A). Furthermore, VR2 specifically recognized the human and wild-type mouse heart VLDL receptor (Human heart, Wild-1/-2). VR2 did not detect VLDL receptor bands in hearts of VLDL receptor (−/−) mice (VKO-1/-2) (Fig. 1B). These results demonstrate that the VR2 is a specific antibody for VLDL receptor of humans and mice.

3.2. Western blot and northern blot analyses of VLDL receptor in macrophages

In detection of VLDL receptors in several macrophage cell lines, although VLDL receptor protein was detected in PMA-treated THP-1 human macrophage cells and wild-type mouse heart, it was not detected in not only cell lines derived from mouse macrophages (Raw 264.7 and J774.2) but also mouse peripheral macrophages (Fig. 1C). In Northern blot analysis, the presence of a strong mRNA band of 3.8 kb in mouse heart was detected. A similarly sized, but much weaker, band was detected in Raw264.7, J774.2 cells and mouse peripheral macrophages. A second, 8.0-kb mRNA band detected in mouse heart was not visible in macrophage cells (Fig. 1D).

3.3. Immunohistochemical analysis of VLDL receptor in WHHLMI rabbit

Next, we examined VLDL receptor localization in rabbits using the VR2 antibody. The VR2 antibody detected rabbit VLDL receptor protein in heart but not in liver by immunohistochemistry (Fig. 2, upper panels). VLDL receptor proteins were clearly detected in some of the RAM11-positive macrophage cells in the thoracic aorta of WHHLMI rabbit, which are indicative of an atherosclerotic lesion (Fig. 2, Aortic lesion-1 and -2). The results were compatible with the Northern blot analysis reported in our previous paper [8,15].

3.4. Immunohistochemical analysis of apoE KO mice and LDL receptor KO mice

In apoE KO and LDL receptor KO mice, the VLDL receptor proteins were detected in the heart but not in the liver (Figs. 3 and 4, upper panels). These results were compatible with Northern blot analysis, indicating that the VLDL receptor is most abundant in heart and is barely detectable in liver [26,27]. In contrast to the atherosclerotic lesion in WHHLMI rabbit thoracic aorta, no VLDL

receptor protein was observed in BM8-positive macrophage cells in aortic atherosclerotic lesions in apoE KO mice (Fig. 3, Aortic lesion-1 and -2) and LDL receptor KO mice whose diet had been supplemented with 1.25% cholesterol for 12 weeks (Fig. 4, Aortic lesion-1 and -2).

4. Discussion

In the present study, we have shown species differences in VLDL receptor protein expression, which suggest that the mechanisms of atherogenesis and growth of atherosclerotic lesions in mouse models may differ from those in human and rabbits.

The rabbit VR2 polyclonal antibody that we raised against the carboxy-terminus of the VLDL receptor protein could recognize VLDL receptor proteins in the heart of human, rabbit, rat and mouse. Our previous and present study also indicated that the VR2 antibody recognized VLDL receptor protein in human macrophage cell lines (PMA-treated THP-1 and HL-60 cells) and in monocyte-derived macrophages [5]. In mouse macrophage cell lines (Raw 264.7 and J774.2) and mouse peritoneal macrophages, however, no VLDL receptor could be detected in Western blot analyses and very low expression of the mRNA in Northern blot analyses was detected. Fujioka et al. [6] showed that the absolute rate of chylomicron remnant metabolism in mouse peritoneal macrophages from VLDL receptor KO mice was similar to that of macrophages from normal mice. Perrey et al. [7] also indicated that in vitro uptake of native apoE-enriched lipoproteins by mouse peritoneal macrophages was primarily mediated by the LDL receptor but not by other apoE-recognizing receptor systems, such as LRP-1 or the VLDL receptor, using mouse peritoneal macrophages from both wild-type and LDL receptor KO mice. Briefly they measured ^{125}I-β-VLDL degradation in mouse peritoneal macrophages from wild type and LDL receptor KO mice. Observed ^{125}I-β-VLDL degradation in the peritoneal macrophages from wild type mice was absolutely abolished in the peritoneal macrophages from LDL receptor KO mice. These studies support our findings that the VLDL receptor proteins were not expressed in mouse macrophages in vitro and in vivo.

Using the VR2 antibody in immunohistochemical studies, we detected VLDL receptor proteins in atherosclerotic lesions in WHHLMI rabbit and that the location of VR2-positive cells was coincident with the location of RAM-11-positive cells (macrophages). These results are in agreement with studies on human atherosclerosis [13,14]. In chow-fed apoE KO or cholesterol-fed LDL receptor KO mice, however, no VR2-positive cells were detected in atherosclerotic lesions, even though mouse heart VLDL receptor proteins were clearly detected by immunohistochemistry using VR2. In studies using VLDL receptor KO plus LDL receptor double-KO mice, the area of the atherosclerotic lesion did not show any significant changes in comparison with that of LDL receptor KO mice [28]. These results suggest that, in mice, VLDL receptor may not play any role in atherogenesis or that VLDL receptors are not expressed in atherosclerotic lesions. Adversely, Van Eck et al. [29] have demonstrated in studies using a technique of bone marrow transplantation to selectively disrupt or reconstitute the VLDL receptor in macrophages in VLDL receptor (+/+) and VLDL receptor (−/−) mice, respectively, that VLDL receptors on macrophages may mediate uptake of atherogenic lipoproteins and play a role in the development of atherosclerosis. However, they did not confirm the VLDL receptor protein in atherosclerotic lesion. It is likely that species-specific differences in VLDL protein expression also exist in other hematopoietic cell types. For example, we previously reported significant expression of splenic VLDL receptor mRNA in rabbits, but a check of the literature indicated that almost all splenic VLDL receptor mRNAs were absent in mice [8,26,27]. Detailed studies of species difference in VLDL receptor expression in hematopoietic cells are required to clarify this matter.

In human, TGRLs have also been isolated from artery segments [30] and it is known that LDL-cholesterol and TGRLs are independent risk factors for CAD. Therefore, we consider that the mechanisms of atherogenesis and/or growth of atherosclerotic lesions may be partly different between mice and humans or rabbits.

In conclusion, since both rabbit and human macrophages express VLDL receptor protein, studies of the importance of VLDL receptor signaling for TGRLs should focus on these species rather than on mouse systems.

Conflict of interest

The authors have no conflicts of interest to disclose.

References

[1] S.M. Grundy, J.I. Cleeman, C.N. Merz, et al., A summary of implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines, Arterioscler. Thromb. Vac. Biol. 24 (2004) 1329–1330.

[2] H.N. Ginsberg, New perspectives on atherogenesis: role of abnormal triglyceride-rich lipoprotein metabolism, Circulation 106 (2002) 2137–2142.

[3] C.K. Glass, J.L. Witztum, Atherosclerosis: the road ahead, Cell 104 (2001) 503–516.

[4] A.C. Li, C.K. Glass, The macrophage foam cell as a target for therapeutic intervention, Nat. Med. 8 (2002) 1235–1242.

[5] S. Kosaka, S. Takahashi, K. Masamura, et al., Evidence of macrophage foam cell formation by very low-density lipoprotein receptor: interferon-gamma inhibition of very low-density lipoprotein receptor expression and foam cell formation in macrophages, Circulation 103 (2001) 1142–1147.

[6] Y. Fujioka, A.D. Cooper, L.G. Fong, Multiple processes are involved in the uptake of chylomicron remnants by mouse peritoneal macrophages, J. Lipid Res. 39 (1998) 2339–2349.

[7] S. Perrey, S. Ishibashi, T. Kitamine, et al., The LDL receptor is the major pathway for beta-VLDL uptake by mouse peritoneal macrophages, Atherosclerosis 154 (2001) 51–60.

[8] S. Takahashi, Y. Kawarabayasi, T. Nakai, et al., Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity, Proc. Natl. Acad. Sci. USA 89 (1992) 9252–9256.

[9] J. Sakai, A. Hoshino, S. Takahashi, et al., Structure, chromosome location, and expression of the human very low density lipoprotein receptor gene, J. Biol. Chem. 269 (1994) 2173–2182.

[10] S. Takahashi, J. Suzuki, M. Kohno, et al., Enhancement of the binding of triglyceride-rich lipoproteins to the very low density lipoprotein receptor by apolipoprotein E and lipoprotein lipase, J. Biol. Chem. 270 (1995) 15747–15754.

[11] S. Takahashi, J. Sakai, T. Fujino, et al., The very low density lipoprotein (VLDL) receptor – a peripheral lipoprotein receptor for remnant lipoproteins into fatty acid active tissues, Mol. Cell Biochem. 248 (2003) 121–127.

[12] S. Takahashi, J. Sakai, T. Fujino, et al., The very low-density lipoprotein (VLDL) receptor: characterization and functions as a peripheral lipoprotein receptor, J. Atheroscler. Thromb. 11 (2004) 200–208.

[13] H.A. Multhaupt, M.E. Gafvels, K. Kariko, et al., Expression of very low density lipoprotein receptor in the vascular wall. Analysis of human tissues by in situ hybridization and immunohistochemistry, Am. J. Pathol. 148 (1996) 1985–1997.

[14] K.M. Argraves, K.F. Kozarsky, J.T. Fallon, et al., The atherogenic lipoprotein Lp(a) is internalized and degraded in a process mediated by the VLDL receptor, J. Clin. Invest. 100 (1997) 2170–2181.

[15] K. Nakazato, T. Ishibashi, J. Shindo, et al., Expression of very low density lipoprotein receptor mRNA in rabbit atherosclerotic lesions, Am. J. Pathol. 149 (1996) 1831–1838.

[16] T.P. Hiltunen, J.S. Luoma, T. Nikkari, et al., Expression of LDL receptor, VLDL receptor, LDL receptor-related protein, and scavenger receptor in rabbit atherosclerotic lesions: marked induction of scavenger receptor and VLDL receptor expression during lesion development, Circulation 97 (1998) 1079–1086.

[17] P.K. Frykman, M.S. Brown, T. Yamamoto, et al., Normal plasma lipoproteins and fertility in gene-targeted mice homozygous for a disruption in the gene encoding very low density lipoprotein receptor, Proc. Natl. Acad. Sci. USA 92 (1995) 8453–8457.

[18] M. Shiomi, T. Ito, S. Yamada, et al., Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit), Arterioscler. Thromb. Vac. Biol. 23 (2003) 1239–1244.

[19] S.H. Zhang, R.L. Reddick, J.A. Piedrahita, et al., Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E, Science 258 (1992) 468–471.

[20] S. Ishibashi, J.L. Goldstein, M.S. Brown, et al., Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice, J. Clin. Invest. 93 (1994) 1885–1893.
[21] T. Yamamoto, C.G. Davis, M.S. Brown, et al., The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA, Cell 39 (1984) 27–38.
[22] D.H. Kim, H. Iijima, K. Goto, et al., Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain, J. Biol. Chem. 271 (1996) 8373–8380.
[23] F.B. Kraemer, Y.D. Chen, R.D. Lopez, et al., Characterization of the binding site on thioglycolate-stimulated mouse peritoneal macrophages that mediates the uptake of very low density lipoproteins, J. Biol. Chem. 258 (1983) 12190–12197.
[24] Y. Zenimaru, S. Takahashi, M. Takahashi, et al., Glucose deprivation accelerates VLDL receptor-mediated TG-rich lipoprotein uptake by AMPK activation in skeletal muscle cells, Biochem. Biophys. Res. Commun. 368 (2008) 716–722.
[25] M. Shiomi, T. Ito, T. Tsukada, et al., Cell compositions of coronary and aortic atherosclerotic lesions in WHHL rabbits differ. An immunohistochemical study, Arterioscler. Thromb. 14 (1994) 931–937.
[26] M.E. Gafvels, L.G. Paavola, C.O. Boyd, et al., Cloning of a complementary deoxyribonucleic acid encoding the murine homolog of the very low density lipoprotein/apolipoprotein-E receptor: expression pattern and assignment of the gene to mouse chromosome 19, Endocrinology 135 (1994) 387–394.
[27] K. Oka, K. Ishimura-Oka, M.J. Chu, et al., Mouse very-low-density-lipoprotein receptor (VLDLR) cDNA cloning, tissue-specific expression and evolutionary relationship with the low-density-lipoprotein receptor, Eur. J. Biochem. 224 (1994) 975–982.
[28] P.J. Tacken, D.J. Delsing, M.J. Gijbels, et al., VLDL receptor deficiency enhances intimal thickening after vascular injury but does not affect atherosclerotic lesion area, Atherosclerosis 162 (2002) 103–110.
[29] M.V. Eck, J. Oost, J.R. Goudriaan, et al., Role of the macrophage very-low-density lipoprotein receptor in atherosclerotic lesion development, Atherosclerosis 183 (2005) 230–237.
[30] J.H. Rapp, A. Lespine, R.L. Hamilton, et al., Triglyceride-rich lipoproteins isolated by selected-affinity anti-apolipoprotein B immunosorption from human atherosclerotic plaque, Arterioscler. Thromb. 14 (1994) 1767–1774.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 406473, 10 pages
doi:10.1155/2011/406473

Review Article

Roles of the WHHL Rabbit in Translational Research on Hypercholesterolemia and Cardiovascular Diseases

Tsutomu Kobayashi,[1] Takashi Ito,[1] and Masashi Shiomi[1,2]

[1] *Institute for Experimental Animals, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*
[2] *Section of Animal Models for Cardiovascular Disease, Division of Cardiovascular Medicine, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*

Correspondence should be addressed to Masashi Shiomi, ieakusm@med.kobe-u.ac.jp

Received 10 September 2010; Revised 17 January 2011; Accepted 15 February 2011

Academic Editor: Andrea Vecchione

Copyright © 2011 Tsutomu Kobayashi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Conquering cardiovascular diseases is one of the most important problems in human health. To overcome cardiovascular diseases, animal models have played important roles. Although the prevalence of genetically modified animals, particularly mice and rats, has contributed greatly to biomedical research, not all human diseases can be investigated in this way. In the study of cardiovascular diseases, mice and rats are inappropriate because of marked differences in lipoprotein metabolism, pathophysiological findings of atherosclerosis, and cardiac function. On the other hand, since lipoprotein metabolism and atherosclerotic lesions in rabbits closely resemble those in humans, several useful animal models for these diseases have been developed in rabbits. One of the most famous of these is the Watanabe heritable hyperlipidemic (WHHL) rabbit, which develops hypercholesterolemia and atherosclerosis spontaneously due to genetic and functional deficiencies of the low-density lipoprotein (LDL) receptor. The WHHL rabbit has been improved to develop myocardial infarction, and the new strain was designated the myocardial infarction-prone WHHL (WHHLMI) rabbit. This review summarizes the importance of selecting animal species for translational research in biomedical science, the development of WHHL and WHHLMI rabbits, their application to the development of hypocholesterolemic and/or antiatherosclerotic drugs, and future prospects regarding WHHL and WHHLMI rabbits.

1. Introduction

According to WHO, the major cause of death within member nations is cardiovascular diseases which account for about 30% of all deaths [1]. This report has indicated that cardiovascular diseases are one of the most important classes of diseases to be overcome. As main risk factors for cardiovascular diseases, hypercholesterolemia, hypertension, disorders in glucose metabolism, smoking, aging, male gender, and social stress are listed. Particularly, control of serum lipid levels is thought to be most important for the prevention of cardiovascular diseases. Currently, in the Japanese population, the upper limits of the normal ranges for serum total cholesterol and LDL cholesterol levels are 220 mg/dL and 140 mg/dL, respectively, and the lower limit of the normal range of HDL cholesterol is defined as 40 mg/dL [2]. According to studies conducted during the 1980s, the incidence of cardiovascular events increases as the serum cholesterol level increases and decreases with hypocholesterolemic treatments [3]. One potent hypocholesterolemic compound is statin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, a rate-limiting enzyme in cholesterol synthesis. The first statin (compactin) was initially developed by a Japanese pharmaceutical company, Sankyo Co. Ltd. [4], and this accelerated the development of cholesterol lowering drugs. The hypocholesterolemic effect of compactin was initially examined with rats. However, the anticipated cholesterol-lowering effect was not observed [5], and the development of this compound was ceased. On the other hand, since compactin showed a potent inhibitory effect on cholesterol synthesis *in vitro* and in chickens, researchers had been looking for other mammalian species applicable for

the assessment of this agent. They found a report of a mutant rabbit strain showing hyperlipidemia, written in a Japanese university's bulletin [6]. This rabbit strain contributed greatly to the development of this compound. The strain was the Watanabe heritable hyperlipidemic (WHHL) rabbit. This was in 1979. Currently, there are seven statins in widespread clinical use. It is estimated that statins are prescribed to more than 40 million patients worldwide and statin therapy has decreased mortality from cardiovascular diseases by 20–50% [7]. Thus statins became essential agents for the treatment of hypercholesterolemia and cardiovascular diseases. These results demonstrate the importance of selecting animal species and/or animal models for translational research to develop therapeutic agents.

This review raises the importance of selecting animal species and/or animal models for translational research by describing the history of the WHHL rabbit and its contribution to studies of hypercholesterolemia and atherosclerosis.

2. The Development of the WHHL Rabbit and Its Characteristics

The history and characteristics of the WHHL rabbit were described in a previous article [8]. In 1973, Dr. Yoshio Watanabe (1927–2008) found one male Japanese white rabbit showing hyperlipidemia. From this mutant, he established a strain, the WHHL rabbit, after seven years of selective breeding. At first, this strain was designated the hyperlipidemic rabbit (HLR) [9]. He submitted a study on this strain to an international journal and renamed it the Watanabe heritable hyperlipidemic (WHHL) rabbit [10], according to a suggestion by the editor.

The strain has 300–700 mg/dL of total cholesterol and 300–400 mg/dL of triglyceride in plasma. There were atherosclerotic lesions in the aorta and xanthoma in the digital joints. The serum glucose level and blood pressure were in normal ranges. In WHHL rabbits, the function of low-density lipoprotein (LDL) receptors on the cell membrane was almost deficient and the clearance of LDL from the circulation delayed [11]. Such symptoms closely resemble human familial hypercholesterolemia (FH), which develops spontaneously, and thus the WHHL rabbit is recognized as the first animal model of this disease. Later, the Nobel Prize winners Goldstein and Brown used WHHL rabbits to verify their hypothesis of an LDL receptor pathway for the metabolism of lipoproteins and clarified human lipoprotein metabolism [12–15]. Their studies revealed that lipoprotein metabolism in the WHHL rabbit closely resembles human FH. Consequently, WHHL rabbits were used as an animal model for the development of cholesterol-lowering agents.

One of the most important features of an animal model for hyperlipidemia is the occurrence of myocardial infarction, the final event of human hypercholesterolemia. The development of severe atherosclerotic lesions in the coronary arteries is a prerequisite for the occurrence of myocardial infarction, but the incidence of coronary atherosclerosis in the WHHL rabbit was initially very low. To establish a new strain which develops coronary atherosclerosis, serial selective breeding was conducted and in 1985, the coronary atherosclerosis-prone WHHL rabbit was developed [16]. Further, a strain with severe coronary atherosclerosis was developed in 1992 [17]. Despite such long-term efforts, the incidence of myocardial infarction remained very low. After a further seven years of selective breeding with improved criteria, such as the use of descendents of rabbits with macrophage-rich coronary lesions, a new strain of WHHL rabbits was established; the myocardial infarction-prone WHHL (WHHLMI) rabbit that spontaneously develops myocardial infarction by progression of coronary atherosclerosis followed by occlusion of the coronary arteries [18]. The characteristics of WHHLMI rabbits are described in a previous review [19]. During their establishment, marked differences in the composition of atherosclerotic plaques were found between the aorta and coronary arteries [20], and the WHHLMI rabbit became an animal model with which to examine the inhibitory effects of drugs on coronary atherosclerosis. These studies suggested genetic factors other than hypercholesterolemia to be important to myocardial infarction and coronary atherosclerosis.

Figure 1 shows the changes in serum lipid levels with aging and the distribution of cholesterol in lipoproteins among WHHLMI rabbits [8]. Serum cholesterol levels are 900–1,400 mg/dL at weaning (3 months old) and at 6 months old, and then decrease gradually (700–1,200 mg/dL at 12 months old, 600–1,100 mg/dL at 18 months old, and 500–1,000 mg/dL at 24 months old). Serum triglyceride levels are 150–500 mg/dL and the change with aging is small. The HMG Co-A reductase activity (cholesterol biosynthesis) in WHHLMI rabbits does not decrease with aging and the precise mechanism of the age-related decrease in cholesterol is still unknown [21]. About 70% of cholesterol occurs in the LDL fraction, 16% in the very low-density lipoprotein (VLDL) fraction, 13% in the intermediate density lipoprotein (IDL) fraction, and 0.8% in the high density lipoprotein (HDL) fraction. Figure 2 shows the extent of atherosclerotic lesions in the coronary arteries and aorta of WHHLMI rabbits [8]. The main coronary artery is the left circumflex artery and the atherosclerotic lesion is more progressed compared to that in the left anterior descending artery and the right coronary artery. Therefore, the degree of coronary atherosclerosis (cross-sectional narrowing) has been evaluated using the left circumflex artery. The degree of aortic atherosclerosis was shown as the ratio of the surface lesion area to the lumen surface area of the aorta. Atherosclerotic lesions develop from 2 months old. At age 12 months, coronary cross-sectional narrowing was about 80% and about 60% of the aortic lumen surface was covered by atherosclerotic lesions. At 18 months old, coronary cross-sectional narrowing and aortic lesion increased to 90% and 80%, respectively [22].

Prior to the development of the WHHLMI strain, WHHL rabbits were used to investigate mechanisms of the development of atherosclerosis, and many aspects have been clarified: accumulation of oxidized LDL in the atherosclerotic lesions [23, 24]; antiatherosclerotic effects of antioxidants (inhibition of oxidized-LDL formation) [25, 26]; the expression of monocyte adhesion molecules on arterial endothelial

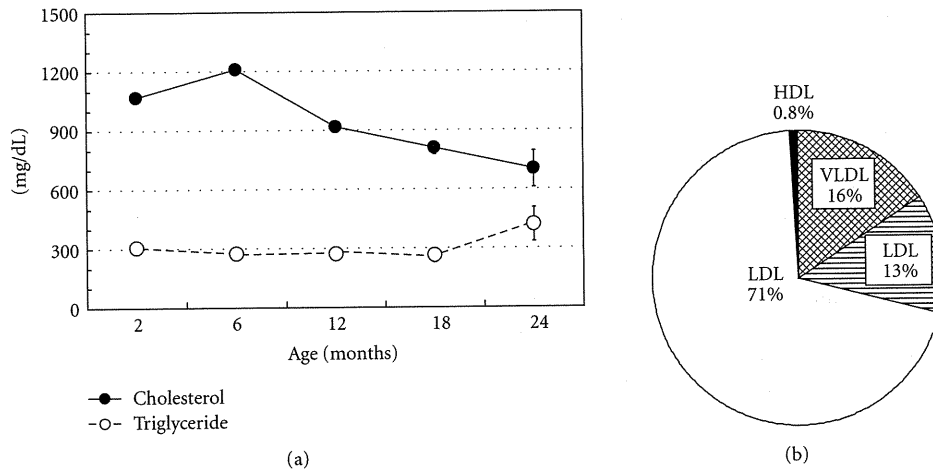


Figure 1: Changes in the serum lipid levels of WHHLMI rabbits with age (a), and the distribution of cholesterol in lipoproteins (b). Data are represented as the mean ± standard error of the mean. The serum cholesterol levels at 12 months old were about 900 mg/dL. Excess LDL cholesterol is atherogenic and HDL has antiatherogenic function. In WHHL rabbits, LDL is accumulated in the plasma and HDL-cholesterol is low, less than 20 mg/dL. The serum cholesterol levels decrease gradually with aging.

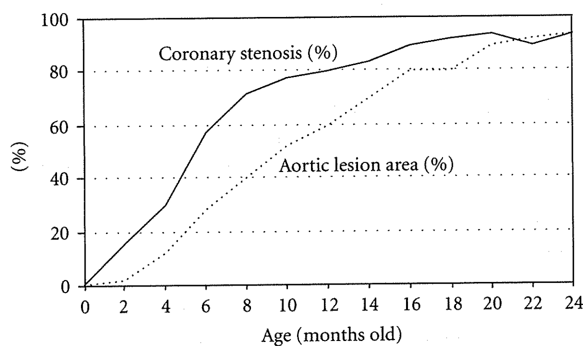


Figure 2: Development of atherosclerotic lesions in WHHLMI rabbits with age. The solid line denotes the degree of coronary atherosclerosis shown as coronary cross-sectional narrowing; lesion areas/area surrounded by the internal elastic lamina ×100 (%). The dotted line denotes the degree of aortic atherosclerosis; sum of the surface areas of the lesion/total surface area of the aortic lumen ×100 (%). Modified from Shiomi and Ito [8].

cells at the initiation of atherosclerosis [27]; scavenging of oxidized LDL at the lesions by macrophages through the scavenger receptors, VLDL receptors, and remnant receptors; accumulation of form cells derived from macrophages in arterial intima followed by further development of atherosclerotic lesions [28–32].

3. Species Differences in Lipid Metabolism and Atherosclerosis

As mentioned, lipoprotein metabolism in rabbits closely resembles that in humans. However, representative laboratory animals such as mice and rats have very different lipoprotein metabolism from that in humans (Table 1). Some examples of major species differences in lipid metabolism are the following. (1) In mice and rats, apoB editing enzyme is observed in the intestine and in the liver, but in humans and rabbits, this enzyme is expressed only in the intestine [33]. In humans and rabbits, apoB-48 is a major apolipoprotein of chylomicron and chylomicron remnants, which carry exogenous lipids derived from foods and apoB100 is a major apolipoprotein of VLDL, IDL, and LDL, which are endogenous lipoproteins derived from liver. In mice and rats, however, endogenous lipoproteins as well as exogenous lipoproteins also contain apoB-48, because of the expression of apoB editing enzyme in the liver [34]. Since the metabolic clearance of lipoproteins containing apoB-48 is very rapid, apoB-48 containing VLDL particles disappear rapidly from the circulation in mice and rats. As a result, the LDL lipid levels in mice and rats are very low compared with those in humans. (2) Hepatic lipase is circulating in the blood stream in mice thus different from humans in degradation of neutral lipids and transportation of free fatty acids into the tissues [35]. (3) In mice and rats, there is no cholesterol-ester transfer protein (CETP) activity in plasma, which transfers cholesterol from HDL to VLDL, IDL, and LDL [36], although CETP plays an important role in humans and rabbits. As a result, in mice and rats, the proportion of cholesterol in the HDL fraction is high compared with other lipoprotein fractions. Therefore, lipoprotein profiles of mice and rats are markedly different from that of humans, even in knockout mice lacking apoE or the LDL-receptors [8]. (4) Competitive inhibitors of a rate-limiting enzyme for cholesterol synthesis, statins, showed potent hypocholesterolemic effects in WHHL rabbits [37–45] but not in mice and rats [5]. In humans, statins are the most effective hypocholesterolemic drugs. These results demonstrate how it is important to choose appropriate species in translational research. (5) C-reactive

Table 1: Comparison of lipid metabolism, atherosclerosis, and cardiac functions between genetically modified mice and WHHLMI rabbits.

	Genetically modified mice	WHHLMI rabbits
Lipid metabolism		
Major lipoprotein in the blood	X (Chylomicron, VLDL)	O (LDL)
Structural protein in theendogenous lipoprotein	X (apoB48)	O (apoB100)
Expression of apoB editing enzyme	X (The small intestine, liver)	O (The small intestine)
CETP activity in the blood	X (No)	O (Exists)
Hepatic lipase	X (Released to circulation)	O (Bound to vessel membrane)
Atherosclerosis		
The coronary lesion	X (Resistant)	Δ (Spontaneously develops)
Composition of the lesions	X (Over accumulation of macrophages)	O (Various lesions)
VLDL receptor	X (no expression)	O (expression)
Heart		
Electrocardiogram		
Limb lead	X (Largely different waveforms)	O (Similar to humans)
Chest lead	X (Difficult to monitor)	O (Similar to humans)
Myocardial ion channel	X (I_{to} and $I_{K,slow}$)	O (I_{kr} and I_{Ks})
Myocardial fibers	X (α-myosin heavy chain)	O (β-myosin heavy chain)
Others		
Inflammatory markers	X (SAP)	O (CRP)
The hypocholesterolemic effect of statins	X (Resistant)	O (Effective)

O: similar to humans; Δ: partly similar to humans; X: largely different from humans.

protein (CRP), a major inflammatory marker in humans and rabbits, which increases in patients with acute coronary syndrome [46], is not responsive to inflammation in mice and rats, due to a lack of complement activation [47]. The major inflammatory marker of mice is serum amyloid P component (SAP), instead of CRP. (6) The types of myocardial fibers in mice are also different from those of humans and rabbits [48]. (7) Moreover, the ECG waveforms in mice and rats are clearly different from those of humans, but rabbit ECG shows similar waveforms to humans [49, 50]. As such, mice and rats have greatly different sets of factors for lipoprotein metabolism and cardiovascular diseases. Therefore, to employ mice and rats for studies on cardiovascular diseases and lipid metabolism, great care is required with analyses and/or the interpretation of the results obtained from experiments.

4. Translational Research on the Development of the Lipid-Lowering Agents

Figure 3 shows features of WHHLMI rabbits which resemble humans and applicable translational research fields. Since the WHHL rabbit is close to humans in lipoprotein metabolism, it was used for the development of various lipid-lowering agents and atherosclerosis-suppressing agents [8]. The hypolipidemic effects of various drugs have been investigated with WHHL rabbits (Table 2): cholesterol synthesis inhibitors, such as HMG-CoA reductase inhibitors and squalene synthetase inhibitors; inhibitors of microsomal triglyceride transfer protein, which works in the assembly of VLDL particles in liver; anionic exchange resins, which block the enterohepatic circulation of bile acids; omega-3 fatty acids, which are a component of fish oil; fibrates, which lower serum triglyceride levels. In studies with a cholesterol synthesis inhibitor, statin, serum total cholesterol levels of WHHL rabbits were decreased dose-dependently by 10–30% compared with the control group [37, 39]. The mechanisms for the reduction in serum cholesterol levels by statins are an increase in expression of mRNA of LDL receptors in the liver [39] and, decrease in the excretion of VLDL cholesterol from the liver in cases of high-dose treatment [38]. The agents that inhibit squalene synthetase, another rate-limiting enzyme in cholesterol synthesis, also decreased the serum cholesterol level by similar mechanisms [51]. Since a small amount of LDL receptor protein can be processed from a precursor to a mature form in WHHL fibroblasts [52], inhibition of cholesterol synthesis in the liver is expected to cause LDL receptors to accumulate on the surface of hepatocytes. Anion exchange resins absorb bile acids at the duodenum and block the enterohepatic circulation [53]. As a result, cholesterol is utilized in the hepatocytes for the synthesis of bile acids, and then the hepatocytes, which was exhausted the cholesterol pool, increase the number of LDL receptor molecules to acquire external cholesterol [39]. Therefore, the combination of an inhibitor for cholesterol synthesis and an anion exchange resin can decrease the serum cholesterol level markedly, and this was proved using WHHL rabbits [40]. Since microsomal triacylglycerol transfer protein (MTP) inhibitors are also effective in WHHL rabbits [54], they may have potential benefit for human FH. The successful treatment in WHHL rabbits means that patients with FH, excluding the LDL-receptor negative type, can be treated with these agents.

Table 2: Drug development using WHHL/WHHLMI rabbits.

	Lipid-lowering effect	Lipid-lowering effect	
		Aorta	Coronary arteries
Cholesterol synthesis inhibitors			
Statins	O	X, O	O
Squalene synthesis inhibitor	O	O	O
Anion exchanger	O	O	
Statins + Anion exchanger	O	O	O
MTP inhibitor	O		
ACAT inhibitor	X, O	X, O	X, O
Antioxidants			
Probucol	O	O	
Vitamin E	X	X, O	
Colony stimulating factor			
MCSF	X, O	O	
GMCSF	X, O	O	
Apo E	X, O	O	
Fibrate	X		
Fish oils, ommega-3 fatty acids	X, O	X, O	
Thiazolidinedione	X	Δ	Δ
Thiazolidinedione + statin	O	O	O
Antihypertensive			
ACE inhibitor	X	O	
AT-II receptor antagonists	X	O	
Calcium antagonists	X	X	
Beta-blockers	X	X	
Gene therapy	O		

O: effective; Δ: partly effective; X: no effect.
Modified from Shiomi and Ito [8].

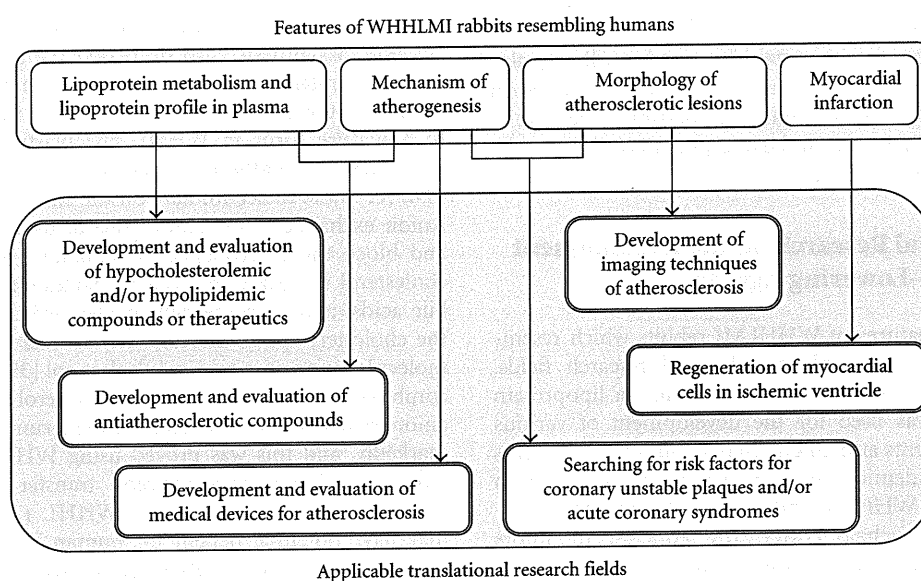


Figure 3: Features of the WHHLMI rabbit resembling humans and applicable translational research fields.