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Coexpression of CLA-1 and Human PDZK1 in Murine Liver Modulates HDL Cholesterol Metabolism

Hidenori Komori, Hidenori Arai, Terumi Kashima, Thierry Huby, Toru Kita, Yukihiko Ueda

Objective—In rodents scavenger receptor class B type I (SR-BI) is a key molecule for selective uptake of cholesteryl ester from high-density lipoprotein (HDL). This study was aimed to clarify the role of the human SR-BI/CD36 and LIMP-II Analogues-1 (CLA-1) as a molecular target of selective uptake of cholesteryl ester from HDL in vivo.

Methods and Results—To clarify the function and regulation of CLA-1 in vivo we produced *CLA-1* BAC transgenic mice. In spite of abundant hepatic RNA expression of *CLA-1*, *CLA-1* BAC transgenic mice had no significant effect on mouse HDL cholesterol. Although coexpression of a human scaffolding protein PDZK1 along with CLA-1 enhanced hepatic CLA-1 expression, it did not affect mouse HDL cholesterol levels, either. However, in the presence of human apoA-1, HDL cholesterol level and size were significantly reduced in CLA-1 transgenic mice, and its reduction was more pronounced in *CLA-1/*human *PDZK1* double transgenic mouse.

Conclusions—We established a mouse model to study human reverse cholesterol transport by expressing *CLA-1*, human *PDZK1,* and human *apoA-I* gene. Our results imply that enhancing CLA-1 expression by human PDZK1 in the liver can modulate HDL cholesterol metabolism and possibly enhance reverse cholesterol transport to prevent the progression of atherosclerosis in human. **(*Arterioscler Thromb Vasc Biol.* 2008;28:1298-1303)**

Key Words: lipoproteins ■ receptor ■ transgenic model ■ apolipoproteins ■ genetically altered mice

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The inverse correlation between plasma high-density lipoprotein (HDL) cholesterol levels and the risk of the coronary heart disease has been established.[1,2] HDL is an antiatherogenic lipoprotein involved in the reverse cholesterol transport (RCT) system where HDL removes excess cholesteryl ester (CE) from peripheral tissues and carries it back to the liver. Therefore, establishing a new therapeutic strategy which can enhance RCT is of great benefit to prevent the development of coronary heart disease.

One of the candidate molecules for RCT is scavenger receptor class B type I (SR-BI). SR-BI was first cloned in 1994 as a modified low-density lipoprotein (LDL) receptor.[3,4] In 1996 this receptor was found to be an HDL receptor that mediates selective uptake of CE from HDL to the liver.[5,6] SR-BI interacts with HDL via apoA-I, which is a major apolipoprotein on HDL molecules,[7] and is necessary for mediating CE uptake.[8,9]

We and others showed that increasing the expression of SR-BI in the liver enhances RCT resulting in a decrease of atheromatous lesion formation in spite of the decreased plasma levels of HDL cholesterol.[10–13] In contrast, decreased expression of SR-BI stimulates the lesion formation in spite of increased HDL cholesterol levels.[14–16] Thus, in rodents SR-BI is a key molecule as an HDL receptor in the liver affecting RCT and the pathogenesis of atherosclerosis. These observations suggest SR-BI as a molecular target for antiatherosclerosis therapy.

However, the RCT system in human is more complicated than in rodents, and it has been hard to study the role of SR-BI in human because of the presence of CE transfer protein (CETP). The human homologue of SR-BI was independently cloned as CD36 and LIMPII analogous-1 (CLA-1).[17,18] CLA-1 functions as a receptor for HDL as well as LDL, VLDL, modified LDL, hepatitis C virus (HCV) in vitro.[19–21] In human CLA-1 is expressed in liver, adrenal gland, testis, and macrophages in the atherosclerotic lesion.[20,22,23] However, the physiological function and the regulation of this molecule still remain unknown.

Tissue expression of SR-BI is regulated both transcriptionally and posttransscriptionally. PDZK1 was cloned as an associating protein with SR-BI stabilizing SR-BI protein on the hepatocyte.[24] PDZK1 is found in liver, kidney, small intestine, pancreas, adrenal cortex, gastrointestinal tract, and testis in human tissue samples.[25] PDZK1 is a 70-kDa protein composed of 4 functional PDZ domains that play an important role in the transport, localization, assembly, and scaffolding of membrane proteins. Coexpression of hamster SR-BI and rat PDZK1 in CHO cells increases SR-BI expression.[24] In

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contrast, hepatic downregulation of PDZK1 with fibrates, MAP17 transgenic mouse, and *PDZK1*-deleted mice showed decreased hepatic SR-BI protein expression posttranscriptionally.[26–28] Interestingly, *PDZK1*-deletion mice show dramatically reduced hepatic SR-BI expression along with increased plasma cholesterol levels.[26,29] Thus in the liver, but not in steroidogenic tissues,[26] posttranscriptional control of SR-BI proteins expression depends on the presence of PDZK1 in rodents.

To explore the function and the regulatory mechanism of CLA-1 in vivo, we generated *CLA-1* transgenic and human *PDZK1* transgenic mouse by introducing human BAC clones. Then we produced double transgenic mouse coexpressing CLA-1 and human PDZK1 to enhance hepatic CLA-1 expression. We investigated CLA-1 expression in the liver along with PDZK1 and their effect on HDL metabolism.

Methods

Bacterial Artificial Chromozome Isolation and Generating Transgenic Mouse

Bacterial artificial chromozome (BAC) clone 265I19 containing full length of the 75-Kb *CLA-1* gene as well as 70-Kb upstream and 25-Kb downstream sequence (Figure 1A) was obtained from Research Genetics by polymerase chain reaction (PCR) screening. BAC was isolated by alkali method and the backbone, pBELOBAC11, was removed by pulsed field gel (PFG) electrophoresis. The linearized fragment was purified by phenol-chloroform extraction and then by dialyzing against an injection buffer (10 mmol/L Tris•HCl pH 7.5; 1 mmol/L EDTA; 100 mmol/L NaCl) through Millipore type VS 0.025-mm membrane (Millipore). Purified DNA was diluted to 5.0 μg/mL for pronuclear injection into FVB mouse fertilized embryos (B.L. Hogan, *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Springs Harbor Express). By the same method the 180-kb genomic DNA fragment containing the full-length sequence of human *PDZK1* with both 34-kbp upstream of exon 1 and 109 kbp downstream of exon 10 (Figure 2A) isolated as BAC clone 354F1 was used to generate human *PDZK1* transgenic mouse.

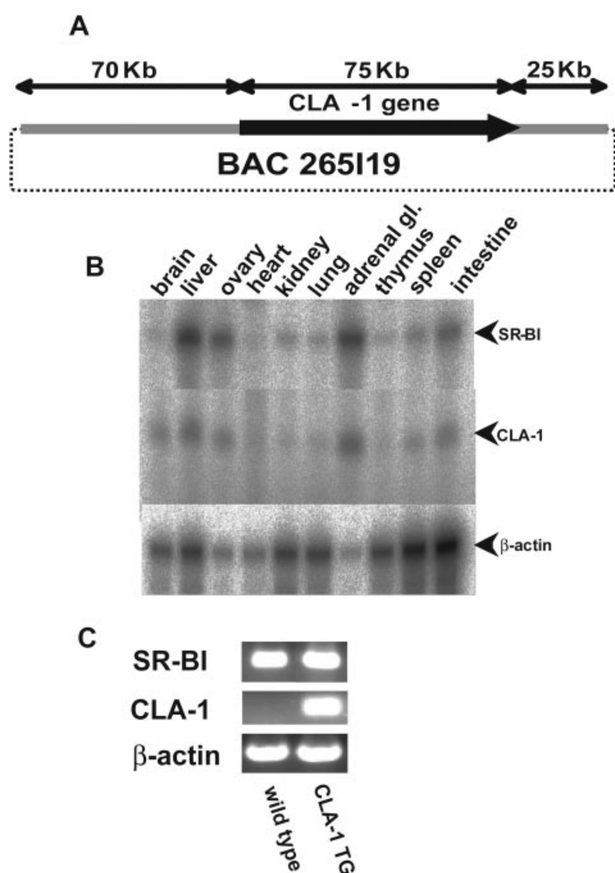

Figure 1. Analysis of CLA-1 BAC transgenic mouse. A, Schema of BAC 265I19 containing the CLA-1 gene. B, RNase protection assay for CLA-1 RNA expression. Ten μg of total RNA in each organ were subjected for the analysis to detect the expression of the first exon of CLA-1 or mouse-SR-BI. C, SR-BI and CLA-1 expression in macrophages by RT-PCR.

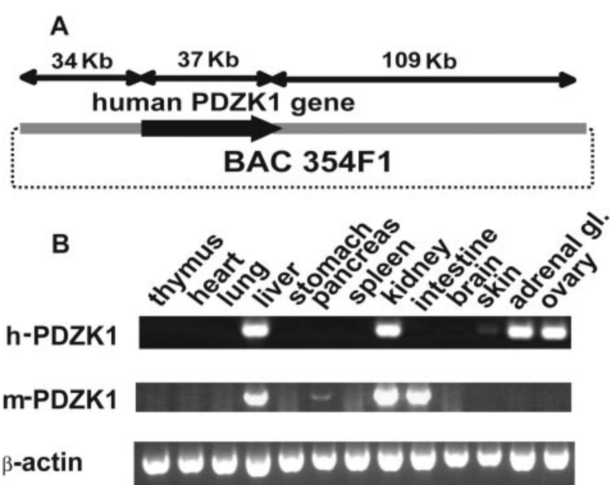

Figure 2. Analysis of human PDZK1 transgenic mouse. A, Schema of BAC 354F1 containing the human *PDZK1* gene. B, Expression pattern of PDZK1 in human *PDZK1* BAC transgenic mouse by RT-PCR. After digestion by DNaseI, 0.5 μg of total RNA were transcribed to subject for PCR amplification. Mouse β-actin was served as an internal control. h-PDZK1 indicates human PDZK1; m-PDZK1, mouse PDZK1.

Other Genetically Engineered Mice

SR-BI–deleted ($SR\text{-}BI^{-/-}$) mouse was generated as described.[30] Human *apoA-I* transgenic mouse ($h\text{-}apoAI^{tg}$)[31] and *apoA-I*–deleted mouse ($m\text{-}apoAI^{-/-}$)[32] were obtained from the Jackson Laboratory (Bar Harbor, Me). All transgenics were established in an FVB strain, and $SR\text{-}BI^{-/-}$ and $m\text{-}apoAI^{-/-}$ mice were back-crossed with FVB mice at least 6 generations, respectively, so that all mice used in the current study have FVB genetic background. All animals in the current study were caged in Kyoto University Animal Facility, which is air-conditioned with controlled light-cycle, and were manipulated along with the Animal Welfare Regulations of Japanese government.

Plasma Lipid and HDL Particle Analysis

At the age of 8 weeks after fed normal chow, blood samples of female mouse were collected by tail vein bleeding in EDTA-coated Microtainer tube (Becton Dickinson and Company). Samples were centrifuged at 7500*g* for 5 minutes and stored at −80°C until analysis. Plasma total (TC) and free cholesterol (FC) was determined by cholesterol oxidase method using Cholesterol E-test Wako (Wako) for TC and Free Cholesterol E-test Wako for FC. Esterified cholesterol (CE) concentration was calculated by subtracting FC from TC. Plasma levels of HDL cholesterol were evaluated by measuring cholesterol after prepicitating the apolipoprotein B containing fraction with 6.5% polyethylene glycol (PEG).[33]

Cholesterol profile in plasma lipoproteins was analyzed by a dual detection high-performance liquid chromatography (HPLC) system with 2 tandem TSKgel LipopropakXL columns according to the method of Usui et al (Lipidsearch System, Skylight Biotech Inc).[34]

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Genotyping of transgenic mice, RNA isolation and analysis, macrophage RNA isolation, Western blotting for liver lysates, and statistical analysis are described under Materials and Methods section in the online Data Supplement available at http://atvb.ahajournals.org.

Results

Gene Expression and Lipid Profile of CLA-1 BAC Transgenic Mouse

To clarify the function of CLA-1 in vivo we generated *CLA-1* transgenic mouse. The inclusion of endogenous regulatory elements within the BAC transgene allows assessment of physiological tissue distribution and regulation of *CLA-1* (Figure 1A). The CLA-1 BAC clone does not contain any other genes. To confirm the expression of the transgene in various tissues of *CLA-1* transgenic mice (*CLA-1 TG*), RNase protection assays were performed (Figure 1B). Under physiological regulation by the human promoter, the expression pattern of *CLA-1* was quite similar to that of the mouse *SR-BI* gene. Expression of the *CLA-1* gene was high in liver, adrenal gland, intestine, and ovary. CLA-1 was also expressed in macrophages of *CLA-1 TG* (Figure 1C).

To investigate the effect of the transgene on lipid metabolism, plasma levels of total cholesterol (TC) and HDL cholesterol (HDL-C) were determined. Plasma TC levels of 10 transgenic mice were not significantly decreased compared with those of the littermate (WT) animals (*CLA-1 TG* 86.5±12.1 mg/dL versus WT 99.5±9.5 mg/dL; n.s.). No significant difference was found in plasma HDL-C levels (*CLA-1 TG* 53.1±23.0 mg/dL versus WT 63.9±10.8 mg/dL; n.s.), either. Although we have established 2 independent transgenic lines, there was no difference in cholesterol levels in both lines. There was no gender difference of cholesterol levels in these mice. Thus CLA-1 expression in addition to endogenous SR-BI did not affect plasma cholesterol levels.

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CLA-1 Protein Expression Was Increased by Coexpression of Human PDZK1 in Liver

Because PDZK1 is an important regulator for SR-BI expression, we assumed that expression of human PDZK1 can be effective for the expression and function of CLA-1. To this end we first produced human *PDZK1* BAC transgenic mouse (*h-PDZK1*). The BAC fragment including the whole human *PDZK1* gene with 34-kb upstream and 110-kb downstream sequences was prepared for microinjection (Figure 2A). To evaluate the expression pattern of the human *PDZK1* transgene and to distinguish it from the endogenous *PDZK1* gene, we performed RT-PCR in various tissues of *h-PDZK1* mice. Similar to endogenous PDZK1 expression in liver, kidney, and intestine, the human *PDZK1* transgene was expressed in liver, kidney, adrenal gland, and ovary (Figure 2B), which is consistent to the previous report.[25] Neither human nor mouse PDZK1, however, was not expressed in macrophages (data not shown).

To assess the effect of human PDZK1 expression on hepatic CLA-1 expression, we then produced *CLA-1/h-PDZK1* double transgenic mouse and performed immunoblotting analysis of hepatic CLA-1 in these mice (Figure 3A). To exclude the effect of endogenous SR-BI and its cross-

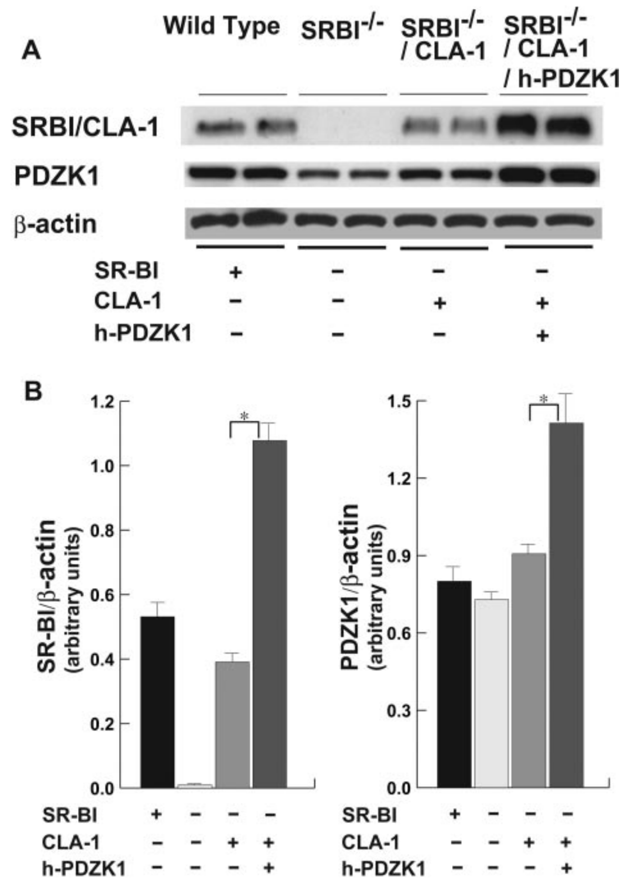

Figure 3. Expression of PDZK1 and CLA-1 in each tissue. A, SR-BI/CLA-1 protein expression in liver. Fifteen micrograms of liver lysates from each 2 mice were subjected for blotting. The PDZK1 band in the h-PDZK1 mouse represents the sum of mouse and human PDZK1. B, Quantitative analysis of SR-BI and PDZK1 protein expression in liver by Optical densitometry. *$P<0.05$.

reaction to the SR-BI/CLA-1 antibody, we introduced the *CLA-1* transgene in *SR-BI*$^{-/-}$ mice, producing *SR-BI*$^{-/-}$/*CLA-1* and *SR-BI*$^{-/-}$/*CLA-1/h-PDZK1* mice. Because the PDZK1 antibody does not distinguish between human and mouse PDZK1, the band corresponding to PDZK1 in *SR-BI*$^{-/-}$/*CLA-1/h-PDZK1* mouse represent the sum of endogenous and transgenic expression. Compared with SR-BI expression in WT mice, *SR-BI*$^{-/-}$/*CLA-1* mouse expressed a significantly lower level of CLA-1 protein in the liver (Figure 3B). However, introduction of the *PDZK1* transgene (*SR-BI*$^{-/-}$/*CLA-1/h-PDZK1* mice) significantly increased expression of human PDZK1 protein along with CLA-1 protein expression. Thus human PDZK1 expression in the liver enhanced hepatic CLA-1 protein expression.

Species Difference in apoA-I–SR-BI Interaction on CE Uptake

To determine whether CLA-1 can regulate HDL-C metabolism in the presence of human PDZK1, we measured cholesterol levels in these mice (Table). HDL-C was increased by approximately 2 folds in the absence of SR-BI or CLA-1 (WT: 96.3±17.3 mg/dL versus *SR-BI*$^{-/-}$: 209.0±17.8 mg/dL; $P<0.05$). However, introducing lower levels of CLA-1 (*SR-*

Table. Plasma Cholesterol Levels in Transgenic Mice

ApoA-I Genotype	SR-BI$^{+/+}$	SR-BI$^{-/-}$	SR-BI$^{-/-}$/CLA-1	SR-BI$^{-/-}$/CLA-1/h-PDZK1
mapoAI$^{+/+}$				
TC	111.3±15.1* (−53%)	234.0±18.8	215.8±20.5 (−8%)	206.7±12.3* (−12%)
HDL-C	96.3±17.3* (−54%)	209.0±17.8	194.7±22.8 (−7%)	185.6±19.3 (−11%)
CE	79.9±6.7* (−35%)	123.7±32.6	143.1±14.0 (+16%)	141.3±14.8 (+14%)
FC	31.4±2.6* (−72%)	110.3±29.1	72.7±7.1 (−34%)	65.0±6.8* (−41%)
mapoAI$^{-/-}$/hapoAI^{tg}				
TC	162.1±16.5† (−72%)	580.2±85.5	359.8±42.5† (−38%)	286.6±32.2† (−51%)
HDL-C	147.9±15.4† (−73%)	557.3±38.3	339.8±36.9† (−39%)	247.4±45.9† (−56%)
CE	114.4±5.5† (−67%)	342.6±90.9	235.2±40.9† (−31%)	179.3±31.2† (−48%)
FC	47.7±2.3† (−80%)	237.6±63.0	124.6±21.7† (−48%)	107.3±18.7† (−55%)

Values are expressed as means±SD (mg/dl).

TC indicates total cholesterol; HDL-C, HDL cholesterol; CE, esterified cholesterol; FC, free cholesterol; mapoAI, mouse apoA-I; hapoAI, human apoA-I.

*$P<0.05$ vs mapoAI$^{+/+}$/SR-BI$^{-/-}$; †$P<0.05$ vs mapoAI$^{-/-}$/hapoAI/SR-BI$^{-/-}$.

(−%) percent reduction compared to the value of SR-BI$^{-/-}$ in each apoA-I genotype.

BI$^{-/-}$/CLA-1) or higher levels of CLA-1 in the presence of PDZK1 (*SR-BI$^{-/-}$*/CLA-1/*h-PDZK1*) did not show any change in HDL-C.

Therefore, we presumed that CLA-1 requires human apoA-I for its sufficient interaction with HDL. To address this issue we introduced human *apoA-I* in the absence of mouse *apoA-I*, producing mouse *apoA-I*–deleted/human *apoA-I* transgenic mice (*m-apoAI$^{-/-}$*/h-apoAI ^{tg}). Introducing human *apoA-I* resulted in a 1.5-fold increase in HDL-C. In the absence of *SR-BI* or *CLA-1*, HDL-C was increased by approximately 3.5 folds (Table). In contrast to the results in mouse *apoA-I*, introducing *CLA-1* lowered HDL-C by 39% (*SR-BI$^{-/-}$/CLA-1*: 339.8±36.9 mg/dL versus *SR-BI$^{-/-}$*: 557.3±38.3 mg/dL; $P<0.05$). Addition of the human *PDZK1* transgene further reduced HDL-C by 56% (*SR-BI$^{-/-}$/CLA-1/h-PDZK1*: 247.4±45.9 mg/dL; $P<0.05$ compared to *SR-BI$^{-/-}$/CLA-1*). We also determined the plasma CE and FC levels in these mice. Plasma CE levels were not different among the 3 groups of *SR-BI$^{-/-}$* mice in the presence of mouse *apoA-I* (Table). However, plasma FC levels significantly decreased in the presence of *CLA-1* and *h-PDZK1*. Meanwhile, in the presence of human *apoA-I*, CE and FC decreased significantly along with HDL-C by the addition of *CLA-1* and *h-PDZK1*. By HPLC analysis the HDL particle size was also decreased along with the expression level of CLA-1 (Figure 4). However, the pattern of HDL particle size is somewhat different in mice with mouse or human *apoA-1*. The HDL particle size is almost the same between *SR-BI$^{+/+}$* and *SR-BI$^{-/-}$/CLA-1/h-PDZK1* mice with human *apoA-1*, whereas the HDL particle size is larger in *SR-BI$^{-/-}$/CLA-1/h-PDZK1* mice than in WT mice with mouse *apoA-1*. Thus, effects of CLA-1 expression on plasma CE/FC and HDL particles size were consistent in mice with mouse or human *apoA-1*. These results suggest that CLA-1 requires human apoA-I to interact efficiently with HDL particles in vivo.

Discussion

In this study we have established a mouse model to study human RCT by coexpressing of *CLA-1*, human *PDZK1*, and human *apoA-I* gene and found that CLA-1 expression plays an important role in plasma HDL cholesterol metabolism in vivo. Therefore, this model would be a useful tool to study the function of human CLA-1 and its effect on HDL metabolism. Because we have used BAC clones for transgenic mice to obtain physiological levels of expression, this could be another advantage for in vivo study.

Upregulating hepatic SR-BI can prevent atherosclerosis by accelerating RCT in rodents. However, RCT is more complicated in human. Human has another mechanism to transfer

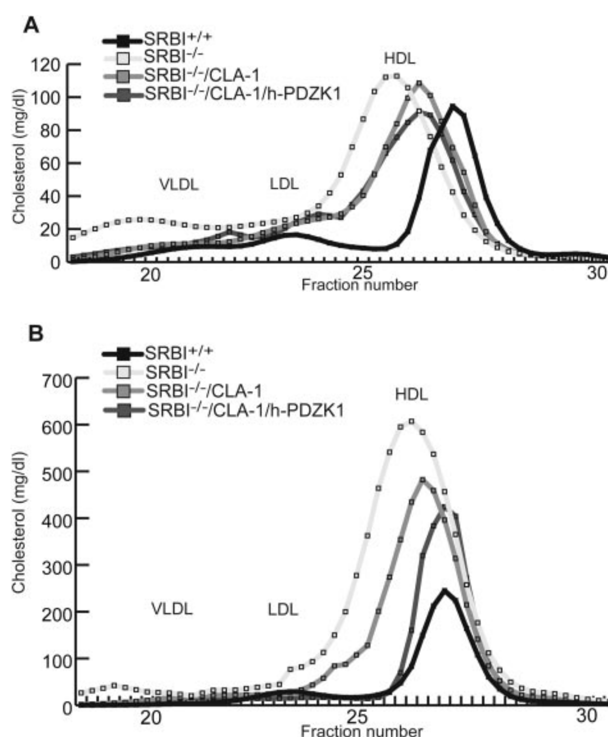

Figure 4. Plasma lipoprotein analyses by HPLC. Plasma samples obtained from 8-week-old chow-fed mice of the indicated genotypes were fractionated by HPLC. A, Plasma obtained from mice with mouse apoA-I. B, Plasma obtained from mice with human apoA-I transgene in the absence of mouse apoA-I (m-apoAI$^{-/-}$/h-apoAI^{tg}).

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CE in HDL particles to the liver, which is mediated by CETP. In CETP-deficient patients,[35,36] HDL-C levels are higher than people with CETP, suggesting that SR-BI–dependent RCT is less important in human lipid metabolism than in rodents. However, there is a huge variability in HDL cholesterol levels in those patients, also suggesting a role of CLA-1 and other molecules in RCT in human. Therefore, by introducing CETP in this model we might be able to produce a model that resembles human RCT.

Ikemoto et al show that PDZK1 can bind its N-terminal PDZ domain to the C terminus of SR-BI in vitro and in vivo and suggest that PDZK1 contributes to the proper sorting and delivery of SR-BI to the plasma membrane in hepatocytes as well as to its stability and function in the liver.[24] We have shown that expression of human PDZK1 enhanced CLA-1 protein expression compared with the mice expressing only CLA-1. Our animal model coexpressing both CLA-1 and human PDZK1 demonstrates that human PDZK1 is an important enhancer of CLA-1 expression in the liver. For the interaction between SR-BI and PDZK1 4 C-terminal amino acids of SR-BI are essential, which are preserved completely in various species.[37] Despite containing the same 4 C-terminal amino acids mouse PDZK1 could not fully stabilize CLA-1 in the liver. These species difference may be caused by a higher constructive change of protein for the molecular interaction. Similarly different SR-BI reaction to the drugs between human and mouse[38] might be explained by the species-specific involvement of PDZK1.

Our data indicate that CLA-1 can selectively uptake CE from HDL containing human apoA-I, suggesting a species difference in apoA-I–SR-BI interaction in the CE uptake mechanism, in spite of its high levels of homology by 80% amino acid identity between mouse SR-BI and CLA-1. It is not clearly known which site of apoA-I is critical for the binding to SR-BI because apoA-I contains multiple regions with amphipatic α-helical repeats, which can bind to SR-BI.[7] Comparing amino acids sequences between human and mouse apoA-I, the carboxyl-terminal region (residue 185 to 243) shows 60% identity between human and mouse, whereas the amino-terminal region (residue 1 to 43) and the central core region (residue 68 to 185) have 72% identity. The carboxyl terminus of apoA-I is involved in protein-lipid interaction, which is critical for the initial rapid binding to HDL, cholesterol efflux from the plasma membrane, and binding to the receptor.[39] Gong et al have reported that the difference of mean hydrophobicity in the helical segment may be responsible for lower efficiency of mouse apoA-I in its stability and lipid binding than of human apoA-I.[40] Thus the constructive difference in the carboxyl terminus may affect the conformation of HDL and contribute to species-specific binding.

We have demonstrated that FC was decreased by the expression of CLA-1 and human-PDZK1, but not CE in mice with mouse *apoA-I*. SR-BI has been reported to mediate not only selective uptake of HDL-CE[5] but also bidirectional transfer of FC between HDL and cells.[41,42] SR-BI–mediated cellular uptake of FC and CE correlates with the expression level of SR-BI in different cell lines.[41] In vivo SR-BI promotes the uptake of HDL-FC and facilitates the rapid clearance of HDL-FC by the liver into bile.[43] However, FC efflux mediated by SR-BI seems to occur independently of acceptor binding to SR-BI.[44] Therefore, it is conceivable that CLA-1 could mediate the uptake of FC from HDL particles independent of species-specific apoA-I. In vitro study is necessary to address this issue.

Hepatitis C virus (HCV) is known to infect hepatocytes via CLA-1. However, mouse SR-BI is not able to bind E2, the envelope glycoprotein of HCV.[21] Because of the species specificity of HCV infection as we found in apoA-I interaction, HCV infection can be studied in vivo only in a chimpanzee model. Therefore, our murine model expressing CLA-1 can be a useful model to study the mechanism of HCV infection.

In conclusion our data implicate that CLA-1 could modulate HDL cholesterol metabolism in human as an HDL receptor in the liver and that CLA-1 and PDZK1 are possible molecular targets for athero-therapeutical strategies in human.

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Disclosures

None.

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