Original Article

Rapid Development of Atherosclerosis in the World’s Smallest Microminipig Fed a High-Fat/High-Cholesterol Diet
A Useful Animal Model Due to its Size and Similarity to Human Pathophysiology

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Aim: Experimental studies of human atherogenesis require an appropriate animal model that mimics human physiology and pathology. Because swine physiology is similar to human physiology, we developed a hyperlipidemia-induced atherosclerosis model using the recently developed world’s smallest Microminipig™.

Methods: These animals weigh only 5 kg at 3 months of age, much smaller than any other miniature pig. We found that the administration of a high-fat/high-cholesterol diet containing at least 0.2% cholesterol without cholic acid for as little as eight weeks induces hypercholesterolemia and subsequent atherosclerosis in these animals.

Results: The serum levels of low-density lipoprotein cholesterol (LDL-C) and the percent distribution of cholesterol in the LDL fractions were markedly increased. The hepatic expression of LDL receptor and hydroxymethylglutaryl-CoA reductase was coordinately decreased. The cholesteryl ester transfer protein activity, which plays a role in reverse cholesterol transport, was detected in the serum of the Microminipigs. Niemann-Pick C1-like 1 protein was expressed in both the liver and small intestine; however, hepatic apoB mRNA editing enzyme was not expressed. As in humans, and in contrast to that observed in mice, most of the hepatic lipase activity was localized in the liver. These results suggest that the hyperlipidemia-induced gene expression profile linked to cholesterol homeostasis and atherogenesis is similar in Microminipigs and humans.

Conclusion: We conclude that the characteristics of the Microminipig, including its easy handling size, make it an appropriate model for studies of atherosclerosis and related conditions.


Key words: Microminipig, Atherosclerosis, Cholesterol metabolism, CETP

Introduction

Atherosclerosis is a predominant risk factor for cardiovascular and cerebrovascular events and is closely related to serious morbidity and mortality in developed nations. The recent westernization of lifestyle in Japan, especially the increased caloric intake from a fatty diet, may account for the increasing inci-
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Aim

The specific aims of the present study were to further expand upon our previous research and detect evidence of close similarities between MMPigs and humans with respect to lipid metabolism and atherosclerosis. We specifically investigated whether HcD alone (with no SC) induces atherosclerosis in the MMPigs and determined the minimum cholesterol content required to cause disease. We performed a histological evaluation of hyperlipidemia-induced atherosclerosis and assessed the expression of genes regulating cholesterol metabolism, including LDLr, class B scavenger receptor type I (SR-BI), hydroxymethylglutaryl-CoA reductase (HMGCR), apoB mRNA editing enzyme catalytic polypeptide 1 (APOBEC-1) and Niemann-Pick C1-like 1 protein (NPC1L1), which is expressed in the mammalian small intestine and liver and is critical for intestinal cholesterol absorption. The activity and localization of cholesteryl ester transfer protein (CETP) and hepatic lipase (HL) in the MMPigs were also evaluated and compared with those observed in humans.

Methods

Animals and Diet

Male MMPigs 3 months of age were maintained in a special room under environmental conditions with a room temperature of 24 ± 3°C, a relative humidity of 50% ± 20% and a 12-hour light/dark cycle. Tap water was available ad libitum, and the animals were provided a special diet on a daily basis. The body weight was measured once a week. All protocols were approved by the Ethics Committee of Animal Care and Experimentation at Kagoshima University and performed according to the laws (no. 105) and notifications (no. 6) of the Japanese Government. This study was also performed in accordance with the animal welfare bylaws of Shin Nippon Biomedical Laboratories Ltd., a facility fully accredited by the Associa-
tion for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and approved by the International Animal Care and Use Committee.

Twenty-two MMPigs were divided into four groups: seven control animals fed a normal chow diet (NcD) and three groups (five animals in each group) fed HcD for eight weeks. The HcD was composed of 12% lard (Miyoshi Oil & Fat, Tokyo, Japan) and 0.2%, 0.5% or 1.5% cholesterol (Wako Pure Chemical Industries, Osaka, Japan) mixed with NcD (Kodakara 73; Marubeni Nisshin Feed, Tokyo, Japan). After eight weeks, all MMPigs were anesthetized and sacrificed via bilateral axillary artery exsanguination.

Blood Pressure

The arterial systolic and diastolic blood pressures were measured at the foreleg with an apparatus meant for human pediatric use according to the Manchette method.

Hematology and Biochemical Analysis

Blood samples were collected once every two weeks for general hematology, biochemistry and lipoprotein profiling. The biochemical parameters measured in the blood samples included the levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transpeptidase, glucose and total bilirubin. The levels of total cholesterol (TC), chylomicrons (CM), very low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were analyzed using an automated agarose gel electrophoresis apparatus (Epalyzer 2, Helena Laboratories, Saitama, Japan).

For measurement of the HL activity, blood was collected from the NcD-fed MMPigs before and 10 minutes after the intravenous injection of sodium heparin (50 unit/kg BW) at 5 and 25 months of age. Serum samples of the pre- and post-heparin fractions were then analyzed using an HL activity assay kit (Progen Biotechnik GmbH, Heidelberg, Germany). The serum CETP expression and activity in the HDL fractions were assayed in the NcD-fed control MMPigs (See Supplemental text).

Serum ApoB Profiles in the CM and VLDL Fractions

The similarity between the human and MMPig plasma proteomes was investigated using a method for cross-species detection of lipoproteins (Supplemental text). In brief, the CM and VLDL fractions were separated via ultracentrifugation. The samples were then alkylated, digested with trypsin and analyzed on a nano HPLC system coupled with a triple quadrupole mass spectrometer.

Evaluation of Visceral and Subcutaneous Fat

The weight of visceral tissue fat was measured at necropsy. The accumulation of subcutaneous fatty tissue (back fat thickness) was evaluated on computed tomography (CT) performed at the beginning and end of the study. The back fat thickness was measured at the midportion of the level between the lower angles of both scapulae, and the percentage increase in thickness after the eight-week dietary treatment was calculated.

Pathological Examination

At necropsy, the aorta, arteries, heart, liver, kidneys, spleen and small intestine were removed from each animal. The heart, liver, kidneys, spleen and visceral (omental and mesenteric) adipose tissue were weighed. All organs were fixed in 10% phosphate-buffered formalin and routinely processed as paraffin-embedded, 5-μm-thick tissue sections stained with hematoxylin and eosin (H&E) and Elastica-Masson stains. The aortas were longitudinally incised and fixed with 10% buffered formalin for 24 hours, followed by staining with Oil-red O stain for an en face analysis. The Oil-red O-stained area relative to the entire surface was calculated using the Image J software program. Immunostaining for atherosclerotic lesions was performed (Envision kit, Dako Cytomation, Kyoto, Japan) on the paraffin-embedded sections using antibodies against smooth muscle actin (anti-α-SMA clone 1A4, ×100; Dako Cytomation) and macrophages (anti-lysozyme rabbit polyclonal antibody, ×2,000; Dako Cytomation).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The liver and small intestine were stored in RNAlater immediately following sample collection, and total RNA was extracted using the mirVana miRNA isolation kit (Invitrogen, Carlsbad, CA). The mRNA expression was quantified using qRT-PCR with a TaqMan quantitative PCR analysis (Applied Biosystems, Oyster Bay, NY). Supplemental Table 1 lists the genes investigated and the primers/probes used for PCR. The primers and probes were either obtained from predesigned gene expression assays or designed based on the sequence information of domestic swine (Applied Biosystems). The expression level of GAPDH mRNA was used as an internal control.
Atherosclerosis in the World’s Smallest Microminipig

In mesenteric adipose tissues were observed. The back fat thickness was significantly increased after eight weeks in the 1.5% cholesterol-fed MMPigs, as compared with that observed in the control MMPigs (Table 1). The blood pressure levels, both systolic and diastolic, were similar in the HcD-fed and NcD-fed control MMPigs.

Hematology and Blood Biochemistry

Considering the normal reference data for MMPigs, no animals exhibited leukopenia, leukocytosis or anemia after being fed NcD or HcD for eight weeks. No increases in the levels of AST, ALT or LDH occurred in either the NcD- or HcD-fed animals. The levels of ALP, γ-GTP and total bilirubin were moderately increased at scattered time points in the animals fed 0.5% or 1.5% cholesterol. No differences in blood glucose were noted between the HcD-fed and NcD-fed MMPigs.

Table 1. Visceral and subcutaneous adiposity in the MMPigs

<table>
<thead>
<tr>
<th>% cho in diet</th>
<th>0</th>
<th>0.2</th>
<th>0.5</th>
<th>1.5</th>
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<tbody>
<tr>
<td>Relative weight (g/kg)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omentum</td>
<td>0.80 ± 0.25</td>
<td>1.78 ± 0.30*</td>
<td>1.96 ± 0.19**</td>
<td>1.78 ± 0.36*</td>
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<tr>
<td>Mesenterium</td>
<td>3.05 ± 0.47</td>
<td>4.30 ± 0.64</td>
<td>4.40 ± 0.35</td>
<td>4.22 ± 0.88</td>
</tr>
<tr>
<td>% increase of BFT</td>
<td>147.7 ± 38.8</td>
<td>214.0 ± 18.8</td>
<td>211.2 ± 26.4</td>
<td>234.8 ± 21.8*</td>
</tr>
</tbody>
</table>

The data are presented as the mean ± SE. cho, cholesterol; BFT, back fat thickness *p < 0.05 and **p < 0.01 vs. the control (0 % cho diet)

Statistical Analysis

All results are expressed as the mean ± SE. The statistical analysis of the differences between groups was performed using Student’s t-test, and the results were considered to be significant at p < 0.05.

Results

Body Weight, Adiposity and Blood Pressure

A 5-month-old male NcD-fed MMPig, 7 kg in body weight, is shown in Fig. 1A. The body weight values increased in all groups during the eight-week experimental period. Compared with the NcD-fed MMPigs, the HcD-fed MMPigs showed a more rapid increase in body weight. In contrast, no significant differences in body weight were observed between the NcD- and HcD-fed groups (Fig. 1B). The omental fat weight was higher in the HcD-fed animals than in the NcD-fed animals; however, no significant differences in mesenteric adipose tissues were observed. The back fat thickness was significantly increased after eight weeks in the 1.5% cholesterol-fed MMPigs, as compared with that observed in the control MMPigs (Table 1). The blood pressure levels, both systolic and diastolic, were similar in the HcD-fed and NcD-fed control MMPigs.

Hematology and Blood Biochemistry

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Serum Lipoprotein Profile

The MMPigs became hypercholesterolemic after two weeks of the HcD at all cholesterol concentrations (0.2%, 0.5% and 1.5% cholesterol) compared with that observed in the NcD-fed controls (Fig. 2A). The TC levels plateaued after two weeks. The VLDL-C and LDL-C levels were increased in the groups fed higher concentrations of cholesterol (0.5% and 1.5% cholesterol; Fig. 2B and 2C), whereas the HDL-C levels increased in all HcD-fed groups during the eight-week experimental period (Fig. 2D). The percent distribution of cholesterol with 0.5% and 1.5% cholesterol loading was increased in the LDL fractions and decreased in the HDL fractions at every time point during the experiment (Fig. 2E and 2F). The serum TG levels were similar in all groups.

Expression of LDLr, SR-BI, HMGCR, SREBP-2 and NPC1L1

The hepatic expression of LDLr and HMGCR
was downregulated in the MMPigs fed HcD for eight weeks at higher dietary cholesterol concentrations (0.5% and 1.5% cholesterol; Fig. 3A and 3B). The expression levels of these two genes were highly correlated with that of SREBP-2, irrespective of diet (Fig. 3C and 3D). The expression levels of SR-BI in the liver were unchanged during HcD consumption (Fig. 4A). The expression of NPC1L1 in the small intestine was not decreased in the jejunum or ileum in the animals fed the HcD compared with that observed in the controls (data not shown); however, the hepatic expression was markedly reduced in the HcD-fed MMPigs (Fig. 4B).

HL and CETP Activity

The HL activity was much higher in the post-heparin fractions vs. the pre-heparin fractions in the NcD-fed MMPigs, thus demonstrating an activity of more than 90% in the circulation following the administration of sodium heparin (Table 2). For CETP, the protein expression, detected using Western blotting with antibodies against human CETP, and activity were detected in the HDL fraction in the serum of the NcD-fed MMPigs (Supplemental Fig. 1).

Expression of apoB mRNA Editing Enzyme and Serum apoB Profile

Hepatic APOBEC-1 is expressed in mice but not in humans or rabbits, and the editing enzymatic action generates apoB48 to form VLDL, including both apoB48 and apoB100. Under our experimental conditions, the APOBEC-1 expression was detected using qRT-PCR in the small intestine alone and not in the liver in the MMPigs (Fig. 4C). We detected apoA1 and apoB48/100 peptides in both the human and swine VLDL and CM fractions (Supplemental Fig. 2).

Hyperlipidemia-Induced Atherosclerosis

The en face analysis demonstrated that aortic atherosclerotic lesions were significantly increased in the HcD-fed MMPigs at all dietary cholesterol concentra-
The atherosclerotic lesions were located in the intima (Fig. 5A) and primarily composed of infiltration of foam cells (Fig. 5B). Elastica-Masson Tri-

Table 2. Plasma hepatic lipase activity in the NcD-fed MMPigs

<table>
<thead>
<tr>
<th>age (month)</th>
<th>25</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>mean ± SE</th>
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<td>animal No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.W. (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-heparin</td>
<td>0.07</td>
<td>0.24</td>
<td>0.08</td>
<td>0.09</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>post-heparin</td>
<td>2.47</td>
<td>2.37</td>
<td>2.66</td>
<td>3.42</td>
<td>2.73 ± 0.41</td>
</tr>
<tr>
<td>liver-bound</td>
<td>2.40</td>
<td>2.13</td>
<td>2.58</td>
<td>3.33</td>
<td>2.61 ± 0.46</td>
</tr>
<tr>
<td>% in liver</td>
<td>97.2</td>
<td>89.9</td>
<td>97.0</td>
<td>97.4</td>
<td>95.4 ± 2.8</td>
</tr>
</tbody>
</table>

NcD, normal chow diet; B.W., body weight; HL, hepatic lipase

Fig. 4. Hepatic expression of SR-BI, NPC1L1 and APOBEC-1 and quantitative analysis of hyperlipidemia-induced atherosclerosis (A) The hepatic expression of SR-BI in the HcD-fed groups was similar to that observed in the NcD-fed controls. (B) The hepatic NPC1L1 expression was markedly reduced by HcD feeding. (C) The APOBEC-1 expression was detected in the jejunum and ileum but not in the liver. The gene expression levels were calculated as the percentage expression over the level observed in the control NcD-fed group. (D) Oil-red O-stained atherosclerotic lesions in the NcD-fed and HcD-fed MMPigs after eight weeks. (E) The number of atherosclerotic areas stained with Oil-red-O stain was increased in the HcD-fed MMPigs. The values are presented as the mean ± SE. *p < 0.05, **p < 0.01 vs. the control. #p < 0.05, ##p < 0.01 between each group.
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Aortic atherosclerotic lesions of an MMPig fed HcD for eight weeks. (A) (B) Atheromatous plaque in the abdominal aorta with intimal infiltration of foam cells (H&E stain). (C) The internal elastic laminae are duplicated (arrowheads) and the plaque is covered with a fibrous cap (arrows; Elastica-Masson Trichrome stain). (D) An advanced lesion exhibits disruption of the internal elastic lamina and stratification of newly formed laminae (arrowhead; Elastica-Masson Trichrome stain). (E) Atherosclerotic lesions in the coronary artery of an MMPig fed HcD for eight weeks. (F) Light microscopy of an atherosclerotic lesion in the left main trunk at low and high power (H&E stain). (G) The plaque lesion consists of the accumulation of foam cells positive for the macrophage marker lysozyme. (H) A small number of intimal cells are positive for α-SMA. Medial smooth muscle cells positive for α-SMA were used as an internal control.

Fig. 5. Atherosclerotic lesions in the aorta and coronary arteries

chrome staining showed duplication and disruption of the internal elastic lamina (Fig. 5C and 5D). The advanced lesions exhibited fibrous cap formation covering the atheromatous plaques (Fig. 5C). The stages of atherosclerosis were varied in the sections examined; however, in general, the abdominal aortas showed a large amount of advanced lesions. The atherosclerotic lesions in the carotid, coronary and femoral arteries were identical to those observed in the aorta (Fig. 5E and 5F). In the coronary arteries, the
proximal portions, located immediately after branching from the aorta, were severely involved in the atherosclerosis; however, a few lesions in the distal and intramuscular arteries were also observed, especially in the higher cholesterol diet-fed groups. The infiltrating foam cells were positive for the macrophage marker (lysozyme) (Fig. 5G). The atheromatous lesions also demonstrated small numbers of α-SMA-positive smooth muscle cells (Fig. 5H).

Histopathology of other Organs
No significant histological changes were observed in the heart, lungs, spleen or kidneys. Mild fatty degeneration was detected in the liver in the HcD-fed MMPigs at 0.5% and 1.5% cholesterol concentrations (data not shown). Despite the presence of atherosclerotic lesions, no HcD-fed MMPigs experienced thrombosis or spontaneous myocardial or cerebral infarction during the eight-week experiment.

Discussion
Diet-Induced Hypercholesterolemia and Atherosclerosis
This study demonstrated the presence of eight-week HcD-induced hypercholesterolemia and atherosclerosis accompanied by moderate visceral and subcutaneous adiposity in MMPigs. After eight weeks of the administration of HcD containing 1.5% cholesterol, the serum levels of TC reached approximately 600 mg/dL and atherosclerotic areas were seen in approximately 50% of the aortas. The relatively low 0.2% dietary cholesterol concentration was sufficient to induce the formation of aortic atherosclerotic areas in 7.5% of the animals, with TC levels of approximately 200 mg/dL. The consumption of dietary SC, which is often administered to mice in order to induce atherosclerosis, was not necessary to enhance the development of hyperlipidemia-induced atherosclerosis in the MMPigs. It is noteworthy that a period of only eight weeks was sufficient to induce atherosclerosis in MMPigs, whereas other miniature pigs require more than three months to develop this condition.

Serum Lipid Profiles
Both the serum LDL-C and HDL-C levels markedly increased after the administration of the HcD, with a greater increase observed in the LDL-C levels. The percentage distribution of cholesterol into HDL-C was therefore significantly reduced, indicating a proatherogenic lipid profile. This paradoxical increase in HDL-C induced by dietary fat has been reported in both humans and rabbits. metabolic studies have shown that the administration of HcD decreases the catabolic rate while increasing the transport rate of apoA-I, resulting in increased HDL-C levels. Similar observations have been made in human apoA-I transgenic mice, in which the administration of HcD increases the HDL-C levels, whereas metabolic turnover studies indicate a decreased catabolic rate and increased transport rate of HDL-cholesteryl ester (CE) and apoA-I. The diet-induced increase in the HDL-C levels may represent an adaptation for enhancing reverse cholesterol transport mediated via the HDL pathway, although the exact mechanism is unclear. The detection of the enzymatic activity of CETP, which regulates a portion of RCT, in the HDL fraction in the MMPigs supports the presence of this possible adaptive mechanism(s).

Serum ApoB Profiles
In this study, the MMPigs, similar to humans and rabbits, did not express hepatic APOBEC-1. The VLDL-C fraction therefore included only apoB100 and not apoB48. In contrast, mice express hepatic APOBEC-1 to generate apoB48-containing VLDL-C and subsequently lower the LDL-C levels. The transfer of the APOBEC-1 gene in the liver in New Zealand White rabbits and Watanabe heritable hyperlipidemic rabbits results in the production of apoB48-containing VLDL-C and a reduction in LDL-C formation. Serum VLDL-C cannot be converted to LDL-C in humans with hypobetalipoproteinemia because it contains truncated apoB50 due to a premature stop codon in the apoB gene. Therefore, the regulation of the size of apoB by hepatic apoB mRNA editing represents a fundamental mechanism for limiting the generation of atherogenic apoB100-containing lipoproteins. The hepatic expression of APOBEC-1 corresponds closely with a low ratio of [VLDL-C + LDL-C] to HDL-C (<0.5) in dogs (0.26), rats (0.41), mice (0.25) and horses (0.44). Mammals that do not express hepatic APOBEC-1 exhibit higher ratios, including humans (1.92), monkeys (0.91) and pigs (1.4). Rabbits (0.32) are an exception to this rule. In this study, the ratio in the NcD-fed MMPigs was 1.31, indicating a proatherogenic lipid profile in this group.

Expression of LDLr, HMGCR and NPC1L1
The blood cholesterol levels are largely determined by LDLr removal mediated by LDLr and cholesterol synthesis via the HMGCR activity in the liver. The hepatic expression of LDLr and HMGCR is coordinately regulated by the sterol regulatory element-binding protein (SREBP)-2 signaling
pathway; the transcriptional activity of SREBP-2 is usually inhibited when cellular cholesterol is abundant. In the present study, the hepatic expression of LDLr, HMGCR and SREBP-2 was correlated and markedly downregulated following the consumption of HcD. The regulation of these genes and the LDL-C-rich lipid profile in MMPigs is therefore very similar to that observed in humans and is in contrast with that observed in mice, in which downregulation gene responses are minimal.

Intestinal absorption and biliary excretion are additional closely regulated mechanisms of cholesterol homeostasis. A recently identified NPC1L1 protein that regulates intestinal cholesterol absorption is highly expressed in the small intestine in various species, including humans, rabbits and mice. The NPC1L1 protein is also expressed in the human liver, where it partially regulates biliary cholesterol excretion; however, it is not expressed in the murine liver. In the present study, the NPC1L1 gene was expressed in both the small intestine and liver in the MMPigs. The regulatory gene mechanisms of the NPC1L1 expression in both organs include the SREBP-2 pathway, suggesting that a high level of cholesterol suppresses SREBP-2-regulatory genes, such as LDLr, HMGCR and NPC1L1. Although the NPC1L1 expression was not clearly reduced following the administration of the HcD, the detection of the expression of NPC1L1 in both the liver and intestine supports the conclusion that the gene expression profile linked to cholesterol homeostasis is very similar between MMPigs and humans.

Expression of the CETP and HL Activity
CETP catalyzes the transfer of CE from HDL to apoB-containing lipoproteins and is considered to be a key protein for reverse cholesterol transport. Humans and animals, including rabbits and chickens, with documented atherosclerosis susceptibility have a higher level of CETP activity than atherosclerosis-resistant animals, such as cats, dogs, rats and mice. The development of atherosclerosis is accelerated by an atherogenic diet when CETP is genetically introduced in mice, which are naturally deficient in CETP. CETP and apoB100 double transgenic mice also show increased levels of atherosclerosis. However, the overexpression of CETP in apoC-III or lecithin cholesterol acyltransferase transgenic mice inhibits atherosclerosis. Assessments of the effects of CETP on atherogenesis in mice are limited because the lipoprotein metabolism in mice differs markedly from that observed in humans. The relationship between the CETP activity and the development of human atherosclerosis is also controversial at present. Patients with coronary heart disease have lower levels of large HDL particles and higher levels of small, dense (sd) LDL particles. CETP inhibitors, which significantly increase the level of large HDL particles and decrease the level of sdLDL particles, are thought to be effective for reducing the development of atherosclerotic cardiovascular disease. In humans, a deficiency of the CETP activity results in increased plasma HDL-C levels with the generation of large CE-rich HDL particles, supporting this hypothesis. Cases of CETP polymorphism or genetic variation, characterized by a diminished CETP activity, are associated with increased levels of HDL-C, a reduced incidence of coronary heart disease and greater longevity. However, other studies have reported an increased incidence of atherosclerotic cardiovascular and cerebrovascular diseases in CETP-deficient human subjects. Furthermore, a recent clinical study showed that the administration of a CETP inhibitor (dalceptripurin) did not reduce the risk of recurrent cardiovascular events even though it increased the HDL cholesterol levels in patients with a recent history of acute coronary syndrome. Whether CETP inhibitors are effective in preventing cardiovascular disease remains a matter of controversy.

Swine express no or very low levels of CETP activity, although they are susceptible to developing atherosclerosis. In the present study, the CETP gene expression was not detected in the MMPigs using RT-PCR with primers designed from human and rabbit CETP cDNA sequences (data not shown). However, the MMPigs expressed CETP-like proteins and the CE transfer activity. Although the presence of a similar CETP gene remains a matter of debate, MMPigs appear to be a useful model for evaluating the relationship between the CETP activity and atherosclerosis.

HL hydrolyzes TG in HDL to convert HDL-2 to HDL-3 and is involved in the conversion of IDL to LDL. In this study, the HL activity in the MMPigs was mostly detected in the post-heparin plasma, similar to findings observed in humans and rabbits. This suggests that most HL activity is localized on the surface of hepatic sinusoidal endothelial cells, in contrast to that observed in mice, in which most of the activity is detected in the circulation. HL- and apoE-deficient mice exhibit a lesser degree of atherosclerosis than apoE-deficient mice, indicating the proatherogenic role of murine HL. In LDLr and murine endogenous HL-deficient mice with the transgenic overexpression of human HL, which can bind to the surface of hepatic sinusoidal endothelial cells, the levels of VLDL, IDL and LDL are decreased, subse-
Table 3. Comparison of lipoprotein metabolism in mice, humans, rabbits and MMPigs

<table>
<thead>
<tr>
<th>Lipoprotein</th>
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<th>rabbits</th>
<th>humans</th>
<th>MMPigs</th>
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<tr>
<td>CETP</td>
<td>HDL-rich</td>
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<td>HL</td>
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<tr>
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<td>Hepatic NPC1L1</td>
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<tr>
<td>Diet-induced atherosclerosis</td>
<td>resistant</td>
<td>sensitive</td>
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CETP, cholesteryl ester transfer protein; HL, hepatic lipase; LDLr, LDL receptor; NPC1L1, Niemann-Pick C1-like 1 protein.
This table was modified from ref. no. 5 by Fan J. and Watanabe T.

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<table>
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<tr>
<th>Lipoprotein</th>
<th>mice</th>
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<th>humans</th>
<th>MMPigs</th>
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<td>CETP</td>
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<td>down-regulated</td>
<td>down-regulated</td>
</tr>
<tr>
<td>Hepatic NPC1L1</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Diet-induced atherosclerosis</td>
<td>resistant</td>
<td>sensitive</td>
<td>sensitive</td>
<td>sensitive</td>
</tr>
</tbody>
</table>

CETP, cholesteryl ester transfer protein; HL, hepatic lipase; LDLr, LDL receptor; NPC1L1, Niemann-Pick C1-like 1 protein.
This table was modified from ref. no. 5 by Fan J. and Watanabe T.

Consequently reducing atherosclerosis. The overexpression of human HL results in the reduction of HDL and IDL in rabbits. The function of HL appears to be affected by the location in which it is primarily localized (the liver or the circulation).

Conclusion

We herein established a novel swine model of hyperlipidemia-induced atherosclerosis in the world’s smallest MMPig, an animal with a cholesterol metabolism very similar to that observed in humans (Table 3). We believe that MMPigs represent a potential alternative animal model suitable for studies of metabolic syndrome because the alteration of lipid metabolism is one of the key events in this condition.

Acknowledgements

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Conflicts of Interest

None.

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**Supplemental Text**

**Materials and Methods**

**CETP Assay**

**Sample Preparation and Western blotting**

For lipoprotein fractionation, equal volumes of plasma samples were pooled from six MMPs and six rabbits fed normal chow. The lipoproteins were fractionated using fast protein liquid chromatography (FPLC) with a Superose 6 10/300 GL FPLC column (Amersham Biosciences, Piscataway, NJ). Diluted plasma and aliquots of the FPLC fractions were subjected to SDS-PAGE and transferred unto PVDF membranes. Immunoblotting was performed as previously described. Antibodies against cholesteryl ester transfer protein (CE) transfer protein (CETP) (mouse monoclonal, designated as “antibody A,” and rabbit polyclonal, designated as “antibody B”) were purchased from EMD Millipore (Billerica, MA) and Abcam (Cambridge Science Park, Cambridge, UK), respectively. The proteins were visualized using a chemiluminescence method (ECL Plus Western Blotting Detection System; GE Healthcare Piscataway, NJ).

**Diagnosis of CETP Deficiency and Determination of the CETP Mass**

A 59-year-old man and 54-year-old woman with high HDL-C levels (172 and 191 mg/dL, respectively) were referred to the National Defense Medical College Hospital. Blood samples were obtained, and the sera were isolated with centrifugation. Genomic DNA was prepared using a commercially available kit (Qiagen, Frederick, MA). The CETP mass was measured using a sandwich ELISA with JHC1 and JHC2, two monoclonal antibodies specific to human CETP, as previously described. Common CETP mutations (an intron 14 splicing defect, Int14 +1 G>A; amino acid substitution from aspartic acid to glycine at position 442, D442G) were identified using an Invader® assay.

**Cloning and Generation of Recombinant Adenoviruses Encoding for Human CETP**

A recombinant adenovirus expressing human CETP (Ad-hCETP) was produced using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Briefly, an entry clone of the Gateway system (Invitrogen) was generated by cloning the open reading frame into a pENTR/D-TOPO vector (Invitrogen) using first-strand complementary DNA derived from human liver RNA (Clontech, Pao Alto, CA) as a template. The specific primers were as follows: forward: 5’-CAC CAT GCT GGC TGC CAC AGT -3’; reverse: 5’-CTA GCT CAA GCT CTG GAG GAA A-3’. An expression clone for the adenoviral vector was then generated via an LR recombination between the entry clone and a pAd/CMV/V5-DEST (Invitrogen), according to the manufacturer’s protocol.

**Supplemental Table 1. Genes investigated and primers used for PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Custom made primers</th>
<th>RefSeq</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLr</td>
<td>Ss03374441_u1</td>
<td>Forward: CCATGGTGATCCATCCAGAT Reverse: AGTTGACAAAAATTCCTCCAGCAGTA</td>
<td>NM_001206354.1</td>
<td>AF065990.1</td>
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<tr>
<td>SR-BI</td>
<td>Ss03391104_m1</td>
<td></td>
<td>NM_213967.1</td>
<td>AF467889.2</td>
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<tr>
<td>HMGCR</td>
<td>Ss03390147_m1</td>
<td>Forward: CCTGTTCGGAGCGAGTCTCTA Reverse: GAAAGAGGAAATAGTCGAGGTA</td>
<td>NM_001122988.1</td>
<td>DQ432054.1</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>Ss03376492_u1</td>
<td>Probe: TGGGAGCCCCAGAGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOBEC-1</td>
<td></td>
<td></td>
<td>XM_003126519.1</td>
<td></td>
</tr>
<tr>
<td>NPC1L1</td>
<td></td>
<td></td>
<td>XM_003134893.1</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Ss03375435_u1</td>
<td>Forward: CCGTGGGAGCGAGTCTCTA Reverse: GAAAGAGGAAATAGTCGAGGTA</td>
<td>NM_001206359.1</td>
<td>AY307771.1</td>
</tr>
</tbody>
</table>

The assay IDs are listed for the predesigned gene expression assay (ABI). The primers and probes for the APOBEC-1 and NPC1L1 analyses were created based on swine sequences.
The recombinant adenoviral plasmid was purified and then transfected into 293A cells. The adenovirus was purified using the Adeno-X Virus Purification Kit (Clontech), after a sufficient cytopathic effect was observed in the cells. The multiplicity of infection (MOI) was defined as the ratio of the total number of plaque-forming units to the total number of cells infected.

**Injection of the Adenoviral Vector and Blood Sampling**

C57BL/6 mice were obtained from Clea Japan (Tokyo, Japan) and handled according to the guidelines of the National Defense Medical College Institutional Animal Care and Use Committee. The mice were injected with Ad-hCETP (5 × 10⁸ pfu) via the tail vein. Five days after injection, the mice were exsanguinated, and blood samples were obtained.

**Determination of the CETP Activity**

The serum CETP activity was determined according to the method of Cheung et al[^3], with a minor modification. In brief, the d>1.125 fraction isolated via ultracentrifugation from pooled healthy human plasma (n=6, fasting) was incubated with ¹⁴C-cholesterol oleate (Perkin-Elmer) at 37°C for 16 hours, after which the 1.125<d<1.21 fraction was isolated via ultracentrifugation. Following dialysis, the HDL fractions were incubated with human LDL isolated from pooled human plasma (n=6, fasting) in the presence or absence of serum obtained from MMPigs, rabbits, mice or humans. Ten hours after incubation, LDL was precipitated by adding heparin (500 U/mL) and manganese chloride (0.2 μmol/L) to the samples. The radiotracer counts in the supernatants were determined using a scintillation counter. The percentage cholesteryl ester transfer was calculated by dividing the differences between the values obtained in the absence and presence of sera by the values obtained in the absence of sera. The data are expressed as the CE transfer relative to the mean value of sera obtained from eight healthy humans.

**MS/MS Analysis of Apolipoprotein A1 and B**

To detect apolipoprotein (apo)A1, B48 and B100 in the plasma, we utilized a HPLC/MS/MS-based method that can be applied across species. A multiple reaction monitoring (MRM) method was developed for apoA1 and apoB48/100[^4]. First, the chylomicron
Supplemental Table 2. MRM transitions for the targeted peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>m/z (precursor, z = 2)</th>
<th>m/z (product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein A1</td>
<td>AKPALEDLR</td>
<td>506.7929</td>
</tr>
<tr>
<td>Apolipoprotein B48/100</td>
<td>GFEPTLEALFGK, YENYELTLK</td>
<td>654.8453</td>
</tr>
</tbody>
</table>

Thermo lens = 120 for all precursor ions. Z = 2 for all precursor ions

Supplemental Fig. 2.

Extracted MRM chromatograms for ApoA1 (AKPALEDLR) and ApoB48/100 (GFEPTLEALFGK, YENYELTLK) from a nanoHPLC-MS/MS analysis of digested human (A) and MMPig (B) plasma fractions, chyomicron (CM) and very-low-density lipoprotein (VLDL).

Results

CETP Assay

Supplemental Fig. 1A shows the cholesterol distribution in the lipoprotein subfractions isolated using FPLC. The lipoprotein profiles of the MMPs were similar to those of rabbits, except for the higher HDL cholesterol levels. We performed a Western blot analysis using the FPLC subfractions to investigate whether the CETP expression was evident in the MMP. Two antibodies against CETP enabled the visualization of bands of similar molecular size in the HDL fractions. (CM) and very-low-density lipoprotein (VLDL) fractions were separated from human and MMPig plasma using the Himac Centrifuge (CP70MX, Himac Centrifuge, Hitachi Medical Systems), according to the manufacturer’s protocol. The samples were alkylated and digested overnight with trypsin and analyzed on a nano HPLC system (Easy-nLC, ThermoScientific) coupled with a triple quadrupole mass spectrometer (Orbitrap Q Exactive, ThermoScientific). The trap column was the EASY column, C18, 0.1 × 2 cm, 5 μm (ThermoScientific), and the analytical column was the Tip column, ODS, 0.075 × 120 mm (Nikkyo Technos). The flow rate was 0.3 mL/min.
respectively (normal range, 1.04-3.55 μg/mL). A genetic analysis revealed that the man carried the homozygous intronic mutation Int14+1 G>A, while the woman carried the Int14+1 G>A/D442G mutation as a compound heterozygote. We also confirmed the absence of the CE transfer activity in the sera of both subjects. Finally, we observed a robust CE transfer activity in the MMPig sera, 1.5-fold higher than that observed in the humans and 0.7-fold lower than that observed in the rabbits, supporting the possible expression of CETP.

Supplemental Fig. 3.
Sequencing of the tandem mass spectrum of (C) a peptide with 506.8 m/z obtained from the tryptic digest of MMPig CM proteins and (D) a peptide with 654.8 m/z obtained from the tryptic digest of MMPig CM proteins.

ously demonstrated in humans) and hamsters (Supplemental Fig. 1B). We next assessed whether sera obtained from MMPigs activated CE transfer from HDL to LDL. We confirmed that the sera obtained from C57BL/6 wild-type mice do not possess a CE transfer activity and that the intravenous injection of Ad-hCETP results in a 2.4-fold increase in the activity compared with that observed in human sera (Supplemental Fig. 1C). To further validate the reliability of our CETP activity assay, we measured the CE transfer activity in the sera obtained from the subjects with CETP deficiency. The CETP mass in the sera of the 59-year-old man (HDL-C, 172 mg/dL) and 51-year-old woman (191 mg/dL) was <0.1 and 0.4 μg/mL, respectively (normal range, 1.04-3.55 μg/mL). A genetic analysis revealed that the man carried the homozygous intronic mutation Int14+1 G>A, while the woman carried the Int14+1 G>A/D442G mutation as a compound heterozygote. We also confirmed the absence of the CE transfer activity in the sera of both subjects. Finally, we observed a robust CE transfer activity in the MMPig sera, 1.5-fold higher than that observed in the humans and 0.7-fold lower than that observed in the rabbits, supporting the possible expression of CETP.
Identification of ApoA1- and ApoB48/100-Specific Peptides and MRM Transitions

Supplemental Table 2 shows the identified peptide sequence along with the corresponding experimentally determined MRM transitions. Supplemental Fig. 2A and 2B show MRM chromatograms extracted from both the human and pig plasma fractions, CM and VLDL. The amino acid sequences for apoA1 and B48/100 in the humans and pigs were obtained with Proteome Discoverer software program using a Swiss-Prot-TrEMBL database. The sequencing of the tandem mass spectrum of peptides with 506.8 and 654.8 m/z from the tryptic digest of porcine CM proteins was AKPALEDLR and GFEPTLEALFGK, corresponding to peptides of porcine apoA1 (Supplemental Fig. 3A) and apoB48/100 (Supplemental Fig. 3B), respectively.

References


