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Effect of CETP on the Plasma Lipoprotein Profile in Four Strains of Transgenic Mouse

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The plasma cholesteryl ester transfer protein (CETP) plays a central role in high-density lipoprotein (HDL) metabolism and reverse cholesterol transport. There are conflicting views regarding whether or not excessive CETP activity is one of the risk factors of atherosclerosis. To study how much effect CETP can have on the profiles of plasma lipoproteins *in vivo,* we produced four strains of transgenic mouse that expressed different levels of human CETP gene. We analyzed seven groups of mice that had different levels of CETP expression. The cholesterol level of HDL, chylomicron (CM) and VLDL, intermediate density lipoprotein (IDL) and LDL were proportionally changed in association with plasma CETP concentrations (2.9 ± 0.6 to 37.4 ± 1.7 μg/ml) in an allelic dose-dependent manner. We further characterized one of the transgenic strains, CETP-4, by optimizing the experimental condition for the mouse model of atherosclerosis, and found that it would be useful for the development of therapeutics against atherosclerosis.

Key Words: **CETP; transgenic mouse; atherosclerosis; HDL; LDL; VLDL; cholesterol.**

Atherosclerosis is one of the most common diseases in western countries. It is believed to be caused by the deposition of cholesterol in the blood vessel intima, followed by macrophage activation (1). HDL is regarded as an anti-atherosclerotic factor because it absorbs cholesterol from the vessels, while VLDL and LDL are believed to be atherogenic factors. Plasma cholesteryl ester transfer protein (CETP) is a 74 kDa glycoprotein expressed in the liver (2, 3). The function of CETP is to transfer cholesteryl ester from HDL to VLDL and LDL, and to return triglyceride from VLDL and LDL to HDL concomitantly. CETP modulates the increase of VLDL and LDL cholesterol level and excessive CETP activity could be a risk factor of atherosclerosis (4, 5). Indeed, several CETP transgenic mouse strains have been produced that showed higher cholesterol levels of VLDL and LDL and lower levels of HDL than normal mice (6–8). However, there has been no detailed analysis of the relationship between CETP activity and plasma lipoprotein profile *in vivo.* So we produced four strains of human CETP transgenic mice (CETP-2, 3, 4, and 5) that showed different expression levels of plasma CETP. Here, we demonstrate their plasma lipoprotein profile. We also characterized the presence of lipid deposits and calcification in CETP-4 mice fed an optimized atherogenic diet. Based on these findings, a possible role of CETP in the development of atherosclerosis is discussed.

Abbreviations used: AI, atherogenic index; CETP, cholesteryl ester transfer protein; CM, chylomicron; HDL high-density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; VLDL, very low density lipoprotein.

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MATERIALS AND METHODS

Isolation of human CETP cDNA. Human CETP cDNA (9) was isolated by PCR with human liver cDNA (Clontech) as templates. After 5-min incubation at 70°C, the phage cDNA library was chilled on ice and used for template in the PCR. PCRs were performed in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol of primers and 2.5 U of rTaq DNA polymerase (TaKaRa Biomedicals) in a volume of 50 μl. After 3 min of denaturation at 94°C, 30 cycles were performed: a 40-s denaturation at 94°C, a 1 min annealing at 55°C, and a 1.5 min extension at 72°C followed by a final extension step at 72°C for 10 min. The primers were 5′-TCATCTCTAACATCATGGCCG-3′ and 5′-GCTAGCTCAAGCTCTGGAGGAA-3′ for the first half of the fragment and 5′-ATCGATGCTGGCTGCCACAGTCCTGA-3′ and 5′-GTACAGCATGCGGGAGTCCC-3′ for the second half of the fragment. The two PCR products were combined at the *Sph*I site to create a full-length human CETP cDNA, which was then inserted into pBluescript SK(+), and its sequences were confirmed.

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Construction of the expression vector and production of transgenics. The full-length CETP cDNA was inserted to the expression vector pHβAPr-1 (10). The resulting clone, pCETP-1, was digested with *Eco*RI and *Nde*I and the 6.3-kb fragment was fractionated in an agarose gel. The pCETP-1 transgene was dissolved in the injection buffer (10 mM Tris–HCl, pH 7.5 and 0.25 mM EDTA) at approximately 10 pg/μl and injected into pronuclei of fertilized eggs from superovulated C57BL/6J (B6) female mice (CLEA Japan). Transgenic founder mice were identified by PCR with primers of 5′-ACCATGCTGGCTGCCACAGTCCTGA-3′ and 5′-CTAGCTCAAGCTCTGGAGGAA-3′, which can amplify the 1228-bp fragment of human CETP cDNA.

Analysis of the expression of human CETP in the transgenic mice. The RNAs were prepared from liver, small intestine, muscle, heart, lung and kidney of transgenic mice by freezing with liquid nitrogen. The tissue was thawed in the presence of ISOGEN (NIPPON GENE), and total RNA was prepared according to manufacturer specifications. First strand cDNA was synthesized using the oligo dT primer or random primer with First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech). Transgene expression was identified by RT-PCR and the primers, 5′-ACCATGCTGGCTGCCACAGTCCTGA-3′ and 5′-CTAGCTCAAGCTCTGGAGGAA-3′, which can amplify the 213-bp fragment of human CETP cDNA.

Measurement of cholesterol and CETP. Four- to six-month-old male mice, fed a standard rodent chow (CRF-1, Oriental Yeast), were anesthetized and bled in the morning through the infraorbital plexus using microcapillary tubes coated with sodium heparin (Terumo Corporation). Plasma total cholesterol (TC), HDL cholesterol (HDL-C) and the amount of CETP were measured. Cholesterol in plasma and other chromatographic samples was measured using a commercially available kit (Boehringer Mannheim) according to manufacturer's instruction.

Plasma CETP was measured with the sandwich ELISA technique using two monoclonal anti-human CETP antibodies, JHC1 and JHC2. These antibodies were produced using Balb/c mice immunized against CETP that was partially purified from lipoprotein-depleted healthy human plasma by Phenyl Sepharose HP column (Amersham Pharmacia Biotech) and Resource Q column (Amersham Pharmacia Biotech) chromatographies. Each antibody inhibits CETP activity, but their inhibitory profiles are different. JHC1 inhibited CETP activity completely in a dose dependent manner, but JHC2 showed about 30% maximal inhibition with a bell shaped inhibition curve. In the ELISA system, JHC1 is used as the capture antibody (1 μg/well), and biotinylated JHC2 as the detection antibody (3 ng/well). Streptavidin-β-galactosidase (GIBCO) and its substrate, 4-methylumbelliferyl-β-D-galactoside (SIGMA) were used as developing reagents. The detection limit of the assay was approximately 1 ng/ml (0.1 ng/well).

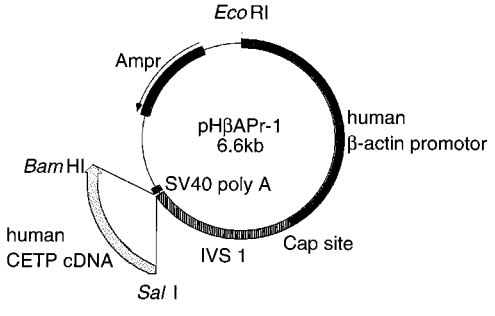

FIG. 1. Construction of human CETP transgene pCETP-1. Human CETP cDNA was inserted to *Sal*I/*Bam*HI site of the expression vector pHβAPr-1. IVS 1, intervening sequence 1; Amp^r, ampicillin resistance; SV40 poly(A), simian virus 40 polyadenylation signal.

TABLE 1

CETP Concentration in Plasma of Human CETP Transgenic Male Mice

Strain		*n*	CETP concentration (μg/ml)
C57BL/6J		7	ND[a]
CETP-2	Homozygote	3	37.4 ± 1.7
	Heterozygote	5	31.3 ± 0.4
CETP-3	Homozygote	4	35.9 ± 2.1
	Heterozygote	7	13.4 ± 0.3
CETP-4	Homozygote	5	19.1 ± 1.3
	Heterozygote	5	9.6 ± 0.3
CETP-5	Homozygote	8	6.1 ± 0.2
	Heterozygote	13	2.9 ± 0.6

Note. CETP concentration expressed as mean ± SD.
[a] ND, not detectable.

Fractionation of lipoproteins from mice plasma by gel filtration. Pooled plasma was obtained from three to nine mice of each strain. An aliquot (200 μl) of pooled plasma was loaded on the two connected Superose 6 columns set in a fast performance liquid chromatography system (Amersham Pharmacia Biotech) (11). Plasma lipoproteins were eluted with 50 mM phosphate-buffered saline containing 1 mM EDTA and 0.02% sodium azide, and fractionated into 0.375 ml aliquots. The cholesterol content in each fraction was measured.

Pathological analysis of CETP-4 strain fed the atherogenic diet. At 8 weeks of age, mice were fed the atherogenic diet(sucrose 50%, fat 5%, cholesterol 0.25%, cellulose 5%, casein 20%, AIN mineral mixture 5%, AIN-76 vitamin mixture 1%, sodium cholate 0.5%, DL-α-tocopherol 0.4%). After exposure to the atherogenic diet for 13 weeks, they were sacrificed and the aortic arches were prepared for histological analysis. The arches were isolated in accordance with the method of Paigen *et al.* (12) and washed with 0.9% saline, fixed with 4% formalin for at least 16-h, then embedded in OCT compound (TISSUE-TEK). Sections were taken every 10 μm from the proximal portion of the aortic sinus. The frozen sections were stained with Oil red O. The size of stained lesion was measured with the imaging analyzer IPAP-WIN (Sumika Technoservice). Calcified lesions were also measured in the same way described above.

RESULTS

We constructed the expression vector of human CETP gene driven by human beta actin promoter (Fig. 1) and introduced it into fertilized eggs of B6 mice. We obtained four strains of transgenic mice, each of which expressed human CETP genes at different levels. All mice developed normally and showed no macroscopic abnormality. ELISA determination of human CETP expression revealed an allelic dosage effect in three of four strains when homozygotic and heterozygotic mice were compared (Table 1). By utilizing seven groups of mice that had different levels of CETP expression, we investigated the cholesterol distribution in plasma lipoproteins by gel filtration (Fig. 2). The highest expresser, the homozygous mice of CETP-2 strain, had markedly decreased HDL cholesterol that was one third of the B6 mice (B6: 70.8 mg/dl, CETP-2 homozygote: 21.7 mg/dl). Among the seven groups of transgenic mice, there was a tight correlation between

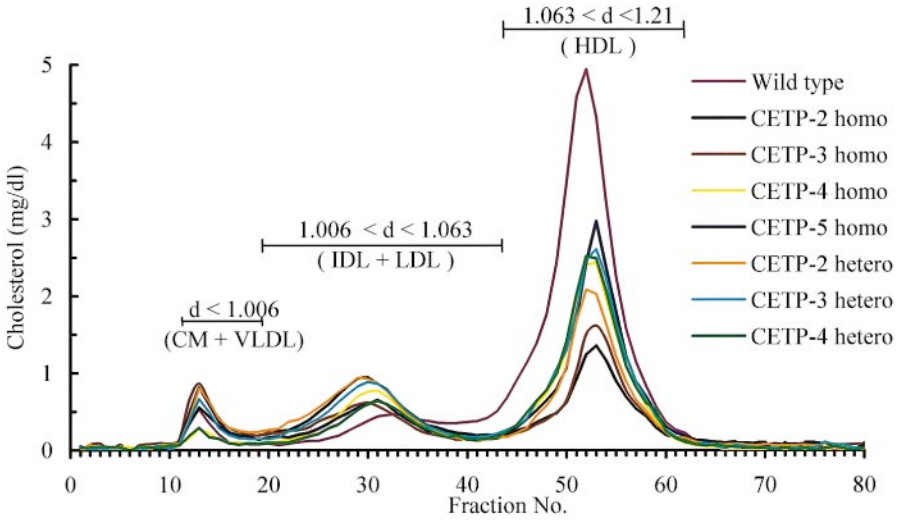

FIG. 2. Cholesterol distribution in plasma lipoproteins in various strains of CETP transgenic mice. Lipoproteins in pooled plasma were fractionated by Superose 6 column and the amount of cholesterol in each lipoprotein fraction was measured as described under Materials and Methods. The eluted positions of chylomicron (CM) + VLDL ($d < 1.006$), intermediate density lipoprotein (IDL) + LDL ($1.006 < d < 1.063$), and HDL ($1.063 < d < 1.21$) were determined with each fraction prepared by ultracentrifugation.

CETP concentration and the cholesterol distribution in plasma lipoproteins such as TC, HDL-C, and in the atherogenic index (AI) (Fig. 3). Notably, the relationship between CETP expression and the ratio of plasma cholesterol in HDL ($r = -0.93$, $P < 0.0025$), IDL + LDL ($r = 0.90$, $P < 0.0045$) or CM + VLDL ($r = 0.70$, $P < 0.04$) was highly significant.

It is well known that B6 strain itself is prone to atherosclerosis. To test if the transgenic mice developed atherosclerosis, we optimized the experimental

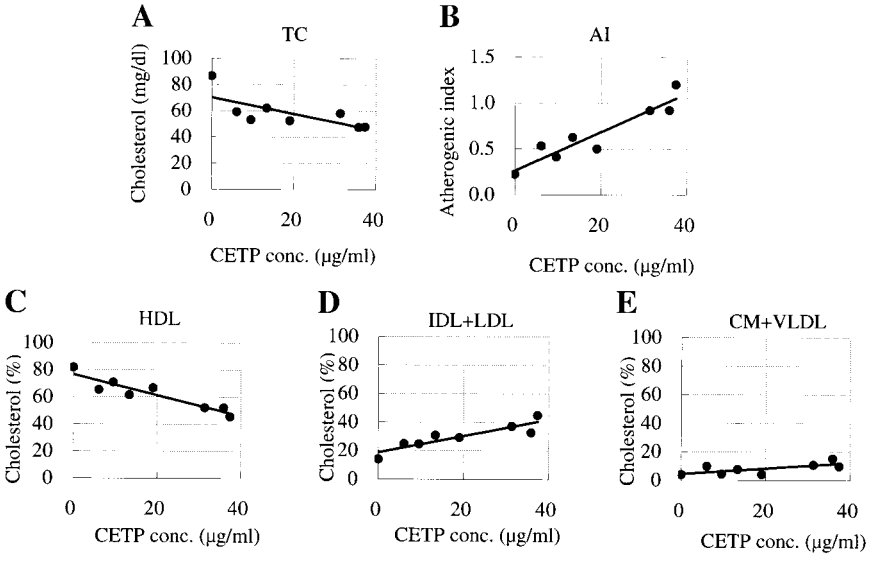

FIG. 3. Relationship between concentration of CETP and the content of cholesterol in plasma lipoproteins observed in CETP transgenic mice. (A) Linear regression analysis demonstrates an inverse correlation between plasma CETP concentration and total cholesterol levels in pooled plasma of B6 (no human CETP) or transgenic mice ($r = -0.72$, $P < 0.003$). (B) Linear regression analysis demonstrates a positive association between plasma CETP concentration and AI of B6 or transgenic mice ($r = 0.94$, $P < 0.0075$). AI was calculated as [{(TC) − (HDL-C)}/(HDL-C)]. (C) Linear regression analysis demonstrates an inverse correlation between plasma CETP concentration and content of HDL-C in TC ($r = -0.93$, $P < 0.0025$). (D) Linear regression analysis demonstrates a positive association between plasma CETP concentration and content of IDL + LDL cholesterol in TC ($r = 0.90$, $P < 0.0045$). (E) Linear regression analysis demonstrates a positive relationship between plasma CETP concentration and content of CM + VLDL cholesterol in TC ($r = 0.70$, $P < 0.04$). Amount of cholesterol in each lipoprotein fraction was determined by gel filtration shown in Fig. 2. TC was the sum of HDL cholesterol, IDL + LDL cholesterol, and CM + VLDL cholesterol. Cholesterol content of each lipoprotein was expressed as the percentage of TC (C, D, and E).

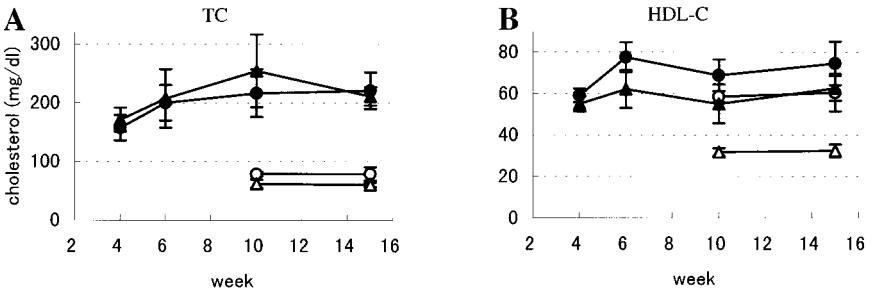

FIG. 4. Effect of atherogenic diet on the plasma cholesterol level in CETP-4 transgenic mice. CETP-4 heterozygote and B6 were fed a control diet, AIN-76 (CETP-4 heterozygote, open triangle; B6, open circle) and atherogenic diet (CETP-4 heterozygote, closed triangle; B6, closed circle). The concentration of TC (A) and HDL-C (B) were measured at 2, 4, 8, and 13 weeks after starting the experiment. The data points with error bars represent the mean ± SD of each group (n = 4–9).

protocol to induce atherogenic lipoprotein profile and atherosclerosis. At first, we had no success following the diet protocol (6) containing 1% cholesterol and 15% fat because the mice lost weight, probably due to a decrease of food intake. Therefore we modified the atherogenic diet according to AIN-76 (13, 14), containing 0.25% cholesterol and 5% fat. After several trials, we found that male mice fed the atherogenic diet for 13 weeks beginning at 8 weeks of age showed the stable atherogenic profile of plasma lipoprotein profile (Fig. 4). After feeding the atherogenic diet, plasma cholesterol in the transgenic mice started to go up rapidly within 3 weeks, then gradually reached a stable level by 9 weeks. We sacrificed the mice at 21 weeks of age, and made histological sections of the aortas to study the atherosclerotic lesions. Distinct deposition of lipid in the intima of aortas was present in six out of nine transgenic mice, while moderate deposition was present in two out of nine B6 mice (Fig. 5). The size of the area stained with Oil red O in lesions of the CETP-4 strain was approximately twice the area found in the B6 mice (Fig. 6A). Furthermore, typical calcification in intima, which was thought to be a progressive atherosclerotic lesion, was detected in CETP-4 heterozygote mice, while we rarely observe these lesions in B6 mice (Fig. 6B).

DISCUSSION

Mice rarely exhibit the lesions of atherosclerosis, but it is believed the genetic background of B6 is a suitable model for its onset (15–21). Therefore, we created four strains of CETP transgenic B6 mice that included seven groups. Each group expressed a characteristic plasma CETP level that correlated with the allelic dosage, homozygote and heterozygote, of the CETP transgene. These four strains of B6 mice proved useful in exploring the dose dependent effect of CETP on

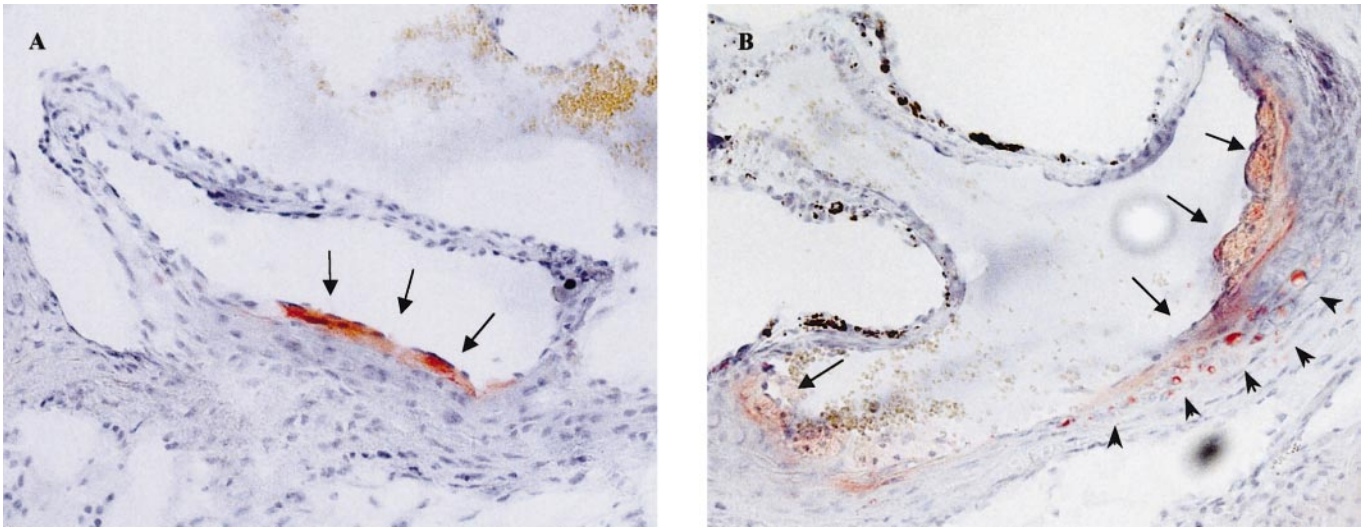

FIG. 5. Typical sections of aortic sinus of mice fed the atherogenic diet for 13 weeks. (A) Section from B6 male mouse. (B) Section from CETP-4 heterozygous transgenic male mouse. Frozen sections of aortic sinus of mice were stained with Oil red O and hematoxylin. The lipid-staining lesions are indicated by arrow in the intima (A) and in the media (B).

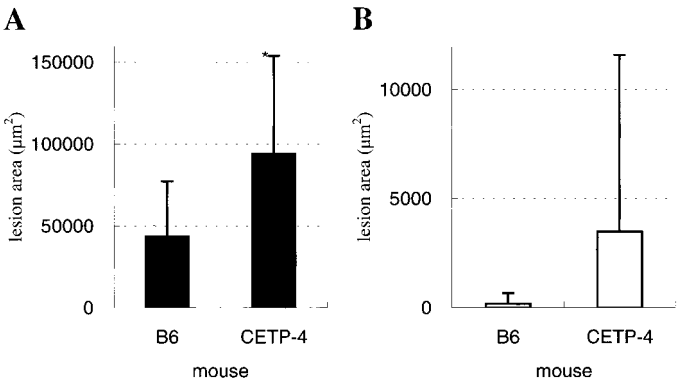

FIG. 6. Comparison of aortic lesion area of lipid accumulation and calcification between CETP-4 transgenic mice and B6 mice. Lesion areas of lipid accumulation (A) and calcification (B) were determined by image analyzer as described under Materials and Methods. The column indicates the mean of approximately 40 cross sections from mice fed the atherogenic diet for 13 weeks. Asterisk, $P < 0.03$ versus the control group.

plasma lipoprotein profile *in vivo*. Some of our transgenic mice had much higher expression of CETP, up to 30 μg/ml, than the transgenic strain previously reported (6, 7). Our strain with the highest level of expression, CETP-2 homozygote, showed the distinct alterations of cholesterol distribution in plasma lipoprotein and AI concomitant with the high level of plasma CETP (Fig. 3). The level of TC or AI was sharply altered even at low levels of CETP expression as in the CETP-5 homozygote, which showed 6.1 μg/ml of human CETP in plasma and about 140% increase of AI compared with B6 (Fig. 3B). This observation is consistent with the previous report that the transgenic mouse, which had about 2.3 μg/ml of human CETP in the plasma, showed about a 40% increase of AI (7). Human plasma CETP level is around 1–2 μg/ml. Because of the steep alteration curve of AI (Fig. 3B), it is conceivable that the level of CETP around 1–2 μg/ml can intervene in the atherogenic environment in human.

There was an inverse correlation between CETP levels and HDL-C. At the highest level of CETP expression, 30 μg/ml in CETP-2 homozygote, the cholesterol level in HDL decreased about 55% compared to that of B6 (B6: 82%, CETP-2 homozygote: 45.4%, Fig. 3C). This occurred with only slight increases in LDL and VLDL cholesterol. We found a high incidence of fatty liver in transgenic male mice (CETP-2, 4, data not shown) as reported previously (22). The decrease of TC observed in the high CETP expresser is probably due to an increase of LDL and VLDL cholesterol uptake in the liver.

In humans, there are variations in CETP levels, probably due to polymorphism of the CETP gene. The relationship between the variation of CETP alleles and atherosclerosis has been controversial until now (23, 24). Chouinard *et al.* reported that the CETP level in plasma was increased in response to food intake or endogenous hypercholesterolemia as a result of increased gene transcription in liver and peripherals (25). Provided that excessive CETP is a risk factor of atherosclerosis, the inhibition of CETP activity would be useful for an effective intervention of the atherosclerosis. We are developing therapeutics which inhibit CETP activity to attenuate atherosclerosis (26). The transgenic mouse that expresses modifiable level of human CETP was thought to be useful for the validation of the drug. Thus a strain of transgenic mouse that shows an atherogenic profile of lipoprotein and, at the same time, develops atherosclerotic lesions in aorta would be preferable for the purpose of drug research and development.

We selected the CETP-4 heterozygote mouse for the pathological experiment because the level of CETP is around 9 μg/ml and seemed to be modifiable with neutralizing reagents at low dosage. The mice were fed the atherogenic diet containing 0.25% cholesterol, 5% fat and 0.5% cholic acid, and showed a more atherogenic lipoprotein profile than those fed a normal diet. Indeed, the pathological examination of aortic arch in CETP-4 heterozygote mice at 13 weeks after exposure to the atherogenic diet showed the distinct lipid deposition and calcification in intima of aorta, which was significant when compared with B6 mice (Fig. 5). The size of the lipid staining by Oil red O in CETP-4 was also twice as much as B6 (Fig. 6).

These results strongly suggest that CETP modulates the cholesterol distribution of lipoproteins in plasma to an atherogenic one and promotes the development of atherosclerosis *in vivo*. We believe that the inhibition of CETP may attenuate atherosclerosis, and we have developed CETP inhibitors that suppress atherosclerosis in rabbit (26). The CETP-4 mouse strain will be helpful for further evaluation of CETP inhibitors and the development of an effective drug against atherosclerosis.

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