A novel swine model for evaluation of dyslipidemia and atherosclerosis induced by human CETP overexpression

Tao Chen¹², Meng Sun¹², Jia-Qiang Wang³, Jin-Jin Cui¹², Zhong-Hua Liu³ and Bo Yu¹²*

Abstract

Background: The mechanism of cholesteryl ester transfer protein (CETP) in lipid metabolism is still unclear. Furthermore, the relationship of CETP and atherosclerosis (AS) has been controversial. As pigs are a good model for both lipid and AS research, we investigated the lipid metabolism of human CETP (hCETP) transgenic pigs and explored the mechanism of CETP in lipid modulation.

Methods: Plasmids expressing the hCETP gene were designed, successfully constructed, and transfected into porcine fetal fibroblasts by liposomes. Using somatic cell nuclear transfer technology and embryonic transfer, hCETP transgenic pigs were generated. After the DNA, RNA, and protein levels were identified, positive hCETP transgenic pigs were selected. Blood samples were collected at different ages to evaluate the phenotypes of biochemical markers, and the metabolomes of plasma samples were analyzed by liquid mass spectrometry.

Results: Eight positive hCETP transgenic pigs and five negative cloned pigs were generated by transgenic technology. Finally, five hCETP transgenic and five cloned pigs were grown healthily. After feeding with a normal diet, hCETP transgenic pigs compared with unmodified pigs had no significant differences in body weight, liver function, kidney function, or plasma ions, while total cholesterol and low-density lipoprotein were higher than in unmodified pigs, and high-density lipoprotein was significantly decreased. Metabolomics analysis showed that there were differences in metabolic components between hCETP transgenic pigs, cloned pigs, and unmodified pigs.

Conclusions: In this study, we created hCETP transgenic pigs that could serve as an excellent model for lipid disorders and atherosclerosis.

Keywords: Cholesteryl ester transfer protein, Transgenic pig, Atherosclerosis, Metabolism

Background

Cholesteryl ester transfer protein (CETP) is a 74-KDa hydrophobic glycoprotein that is secreted mainly from the liver and circulates in plasma. It is present in humans, rabbits, hamsters, chickens, and primates, but absent in rodents, pigs, cows, dogs, and horses [1]. CETP plays a critical role in lipid metabolism in humans, especially in mediating the exchange of cholesteryl esters (CE) and triglycerides (TG) between apoB-containing lipoproteins and high-density lipoprotein (HDL) [2]. The overall role of CETP in atherosclerosis is complex, and whether CETP is an antiatherogenic or proatherogenic protein has been debated for many years. The results of introducing the CETP gene into rodents, which are naturally CETP-deficient, showed reduced HDL levels and severe atherosclerosis (AS) development [3–6]. Rabbits are highly susceptible to developing diet-induced AS because of their naturally high levels of CETP. In addition, it was reported that inhibiting CETP in a rabbit model of AS results in a marked reduction in AS [7]. Furthermore, genetic variants of CETP in humans with low CETP activity may be protective against cardio-vascular disease (CVD), since they exhibit higher HDL levels and reduced low-density lipoprotein (LDL) levels [8]. CETP inhibitors have been a hot topic of CVD research in
recent years. To date, several CETP inhibitors have been brought to clinical trials. Although some CETP inhibitors have proven disappointing, some have been shown to induce significant changes in lipid profiles and metabolism, which permits some optimism for their role in cardiovascular risk reduction [9]. Therefore, more clinical and experimental data is needed to fully understand CETP and substances that affect it.

The majority of animal models used in research are rodents and rabbits whose physiological systems are significantly different from humans. Moreover, the small size of these animals precludes research in intravascular devices and is a challenge for noninvasive imaging. Many important progressive features of human atherosclerosis, such as plaque angiogenesis, plaque ruptures, and thrombosis, are rare or absent in rodent models [10]. Therefore, in recent years, transgenic pigs have been considered as a promising model for scientific research with the sequencing and annotation of the pig genome, given that their genome organization, anatomy, physiology, pathology, body weight, and lifespan closely resemble those of humans [11]. Because studies looking at CETP expression on lipid metabolism and atherosclerosis have produced contradictory results, generating a new transgenic pig model will be helpful for further CETP-related research.

In the present paper, we created such a genetic model by liver- and intestine-specific overexpression of the hCETP gene in pigs. As the pigs aged, this model developed increased plasma total cholesterol (TC) and LDL levels, and decreased plasma triglyceride (TG) and HDL levels. Metabolomics showed some key substances were associated with CETP function in lipid metabolism.

Methods
Ethics statement
All animal care and experiments in this research followed the guidelines of the Second Affiliated Hospital of Harbin Medical University and were approved by the Animal Use and Care committee. All animals (pigs) involved in this research were raised and bred following the guidelines of the Animal Husbandry Department of Heilongjiang, P.R. China.

Genetic constructs
The plasmid pEGFP-C1 (Clontech, CA, USA) was used as a skeleton. We directly subcloned human CETP, a 1.48-kb fragment with NheI/BamHI sites, from cDNA purchased from Sino Biological Inc. China (Catalog Number: HG13276-G). A 1.4-kb fragment of the human ApoC3 promoter was cloned from human genomic DNA with Asel/NheI sites as previously described [6]. The liver-specific expression promoter to drive the targeted hCETP gene and internal ribosome entry site (IRES) were inserted downstream of hCETP, followed by enhanced green fluorescence protein (EGFP) and an SV40 poly-A tail. Finally, the pApoCIII-hCETP-IRESGFP-SV40polyA gene construct was obtained whose structure is shown in Additional file 1: Figure S1-A. Primers used in PCR were as follows: human ApoC3 (Forward: 5′-ATTAA TATTCTGAGGGCAGAGCG-3′; Reverse: 5′-GCT AGCCAGCTGCCCTTAGGG-3′); hCETP (Forward: 5′-GCTAGCATGCTGGCTGAC-3′; Reverse: 5′-GGATCCCTAGCTCAAGCCTGGAG-3′).

Generation of hCETP transgenic pigs
Fibroblast cells derived from E32 fetuses were transfected by the liposome-mediated plasmid pApoC3-hCETP-IRESGFP-SV40polyA, which was based on random insertion of nonhomologous DNA vector into the host genome. After G418 selection, surviving cells were propagated and confirmed by PCR as shown in Additional file 1: Figure S1-B; these were used as nuclear donors, and nuclear transfer was performed as previously described [12]. The reconstructed oocytes were activated and cultured for 18–22 h, and the ones in a good growth state were surgically transferred into an oviduct of the surrogate. The surrogates were kept in a conventional environment for housing pigs. Pregnancies were confirmed by ultrasonography on day 28, and all of the transgenic piglets were delivered by vaginal birth 24 h after induction with prostaglandin.

Identification of hCETP transgenic pigs
DNA analysis
Each DNA sample was cleaved with EcoRI and NheI (TaKaRa, Dalian, China), which can digest the pig genome efficiently. Identification of hCETP transgenic pigs in DNA level was done using primers by PCR. The sequences of the primers were 5′-GAGCAAGGCGAGA GCTTTCA-3′ (forward) and 5′-TGAGAAATTCGAA GCTTTGAGG-3′ (reverse).

Real-time PCR and western blot analysis
To examine the expression of hCETP in transgenic pig tissue, total RNA was isolated from 12 different tissues in two piglets. All samples were processed in triplicate, and the relative expression was standardized to GAPDH. Primers for the hCETP gene were 5′-CCTGACTGCT ACCGTCTTTC-3′ (forward) and 5′-TCCTTCCAG GACCAGCTTAC-3′ (reverse). Total proteins were isolated from plasma of pigs No. 1, 2, 3, 4, 5, 8, 10, 11, 12, 13 for detecting CETP expression. The detailed procedures of RT-PCR and western blotting were performed as previously described [12].
Animals and diets
The pigs were weaned at 28 days and fed a standard diet. All big white pigs were obtained and housed at the Northeast Agriculture University Research Institute (Harbin, China). Pigs had access to autoclaved water and normal diet (Animal husbandry of YuanDa, Harbin, China) ad libitum. Body weight was measured every month.

Plasma analysis
Blood samples were drawn from jugular veins into EDTA-coated tubes after an overnight fast at the time points indicated. Then they were stored on ice and centrifuged within 1.5 h at 1800 rpm for 10 min at 4 °C. All samples were stored at −80 °C until analysis. Plasma TC, HDL, LDL, TG, glucose, liver function, and renal function were measured according to standard laboratory procedures of the Second Affiliated Hospital of Harbin Medical University.

Metabolic analysis
Sample preparation
Before RRLC-QTOF/MS analysis, the plasma and QC samples were thawed and refrozen in a 4 °C water bath. A volume of 1500 mL of methanol was added to 300 mL of plasma. After vortexing vigorously for 2 min, the mixture was allowed to settle at 20 °C for 10 min, and then centrifuged at 14,000 g for 15 min at 4 °C. The supernatant was transferred to a clean vial and dried under nitrogen at 37 °C. The residue was dissolved in 300 mL of acetonitrile–water (3:1, v/v), kept at 30 °C for 10 min, and then vortex mixed for 60 s. The supernatant was then placed into the sample vial for RRLC-QTOF/MS analysis [13].

Liquid chromatography mass spectrometry
A 10 mL aliquot of the pre-treated sample was injected into a 3.0100 mm (1.8 mm) ZORBAX SB-C18 column (Agilent Technologies, Santa Clara, CA, USA) using a rapid resolution liquid chromatography system (1260 series, Agilent Technologies). All samples were maintained at 4 °C during the analysis [14]. Mass spectrometry was performed on an Agilent 6530-QTOF (Agilent Technologies) equipped with an electrospray ionization source operating in electrospray positive (ESI+) and electrospray negative (ESI−) modes. The detailed methods were as previously described [15].

Statistical analysis
The experimental data of each group were expressed as means ± standard deviation. Student’s t-test was applied to determine the differences between groups, and statistical significance was set at P < 0.05. Statistical analysis was performed using SPSS13.0 software.

Results
Production of hCETP transgenic pigs
We obtained a total of 3 litters of pigs from 8 embryo transfers (as shown in Fig. 1a). In one litter, one female pig (No. 1) was born alive. In another litter, 9 female piglets (from No. 2 to No. 10) were born. However, 3 of them died due to deformities or developmental defects and 2 (No. 6 and No. 7) were so weak that they were euthanized to obtain tissues to measure the expression of CETP in vivo. In the last litter, 3 female piglets (No. 11 to No. 13) were born and thrived well.

Identification of hCETP transgenic pigs
Tissue samples of each piglet were taken and DNA was extracted, and the DNA level was identified by PCR. The results showed that pigs No. 2, 3, 5, 7, 9, 11, 12, and were positive hCETP transgenic pigs, and No. 1, 4, 6, 8, 10, and 13 were negative cloned pigs (as shown in Fig. 1b).

RNA was extracted from 12 tissues of piglets No. 6 and No. 7 and detected by real-time PCR. The expression of hCETP was not detected in the liver of piglet No. 6, while the liver of piglet No. 7 had high expression (Fig. 1c). The expression of hCETP in the tissues of piglet No. 7 (Fig. 1c) was consistent with theoretical levels, and hCETP expression in the liver and intestines of the piglets was the highest. The ApoC3 promoter successfully drove the specific expression of the target gene in the liver and intestine. Western blotting also identified that hCETP was expressed in the plasma of No. 2, 3, 5, 11, 12 (Fig. 1d).

Animal characteristics
The body weights of the hCETP transgenic and unmodified pigs showed an upward trend with age as shown in Additional file 1: Figure S1-C. However, there was no significant difference between hCETP transgenic pigs and unmodified pigs at each month of age (P > 0.05).

The plasma samples from hCETP transgenic, cloned, and unmodified pigs were subjected to analysis. Each group contained five pigs. The lipid profile is shown in Fig. 2a-d. At 2 months of age, the lipid profile was similar between hCETP transgenic and unmodified pigs, which showed no significant differences in plasma TC, TG, LDL, and HDL levels (P > 0.05). However, TC and LDL levels were increased in hCETP transgenic pigs compared with unmodified pigs at 5 months (P < 0.05). And HDL levels were decreased in transgenic pigs (P < 0.05). However, there was still no significant difference between these two groups in TG levels (P > 0.05).

Results showed that there were no significant differences in blood glucose levels of hCETP transgenic, cloned, and unmodified pigs (P > 0.05) (Fig. 2e). Moreover, liver and renal function of hCETP transgenic and cloned pigs were normal (Fig. 2f-i). This indicated that the process of human gene CETP integration into the
pig genome did not affect the metabolism of glucose or liver and renal function.

**Plasma metabolic profiling**

The RRLC-QTOF/MS chromatograms for hCETP transgenic, cloned, and unmodified pigs are shown in Fig. 3 and Additional file 1: Figure S2, which indicate peak distributions in positive mode (ESI+) and negative mode (ESI−). From these two mode analyses, we found that these spectra include thousands of plasma metabolites, and there were overlapping peaks among the 3 groups. Moreover, excellent separations among these 3 groups were analyzed, and the results indicated that their plasma metabolites were significantly different from each other.

After pretreatment of the data by XCMS software, PCA analysis was used to observe the components classification trend of transgenic, cloned, and unmodified pigs in the ESI+ mode. The classification effect of PCA worked well and showed that there were significant differences between genetically modified pigs (including hCETP transgenic pigs and negative cloned pigs) and unmodified pigs. However, this plot could not classify hCETP transgenic pigs and cloned pigs. PCA analysis correctly displayed the different components attribution in cloned and unmodified groups (Fig. 4a). Similar to ESI+ mode, PCA analysis in ESI− mode could also distinguish the components of cloned and unmodified pigs (Fig. 4b).

In order to observe the classification between the two groups, we used the supervised learning method PLS-DA to further analyze the data. We chose the modeling component
to construct the PLS score plot (as shown in Additional file 1: Figures S3 and S4). The results showed that the PLS-DA method could clearly show the classification of CETP transgenic, cloned, and unmodified pigs. There was no overlap among the 3 groups in the PLS-DA plot, which indicated that there was a significant difference between any two groups. For evaluating the effect of the PLS-DA model, we calculated $R^2$, $R^2_Y$, and $Q^2$. These parameters indicate the fit and prediction ability, respectively [16]. The results revealed that the PLS-DA model was valid.

**Discussion**

Whether results from rodent research can be directly translated into human clinical trials is still debated. Large animal models that recapitulate human disease pathophysiology are thus attractive to researchers. The rapid development of genetic engineering technologies offers the possibility to do genetic modification in large animals. Here, we created hCETP transgenic pigs that overexpress CETP in a liver- and intestine-specific manner. In order to overexpress CETP specifically in liver and intestine, similar to the human expression profile, we chose the tissue-specific promoter ApoC3, which has been confirmed by Herrera et al. [6]. The present study using mass spectrometry metabolomics showed that the plasma metabolite profile of hCETP transgenic pigs differed from cloned pigs and unmodified pigs.
It has been revealed that CETP plays a physiological role in modulating vascular lipoproteins. Previous human genetic research and animal studies have concluded that CETP is proatherogenic, and inhibiting its activity should reduce CVD risk [17]. However, some studies of genetic CETP deficiency do not show a strong association with CVD risk [18, 19]. A genome-wide association study has identified 6 new loci associated with CVD, which included the CETP gene [20]. Our study also showed that CETP should be proatherogenic, because along with the increase of age, TC and LDL levels of hCETP transgenic pigs were increased and significantly higher than those of normal pigs. However, changes of blood lipid levels in hCETP transgenic pigs were not as significant as those of CETP transgenic mice or rats [3, 6]. One reason for this result may be the low copy number of foreign CETP genes integrating into the genomes of pigs due to using a plasmid vector by liposome transfection [21]. Another is that all generated hCETP transgenic pigs were heterozygotes produced by somatic cell nuclear transfer [22]. If homozygotes of hCETP transgenic pigs were generated after mating with each other, CETP gene expression could be enhanced. Pigs can develop AS under natural conditions, but require longer cycles. While high-fat and high-cholesterol diet feeds can accelerate AS formation, the higher economic costs limit the application of this method [23, 24]. In the last few decades, rabbits have been the main model of AS research for their extreme sensitivity to cholesterol, with CETP being highly expressed and AS plaques forming in a short time [25]. The greatest potential advances of genetically modified pigs in cardiovascular disease studies are that they could mimic human disorders and elucidate disease etiology. The heart, coronary vasculature, and blood flow of pigs is very similar to those of human beings, and overall, pigs can be used for drug, stent, or related interventional research, which can be detected by intracoronary imaging such as intravascular ultrasound (IVUS) or optical coherence tomography (OCT) [26–29]. The successful generation of hCETP transgenic pigs has laid the foundation for an excellent model to decipher the mechanisms of initiation and progression of AS.
The metabonomics of transgenic pigs was analyzed by liquid phase mass spectrometry. After two methods of analysis, the results showed that hCETP transgenic positive, cloned, and unmodified pigs showed significant differences between the three metabolic components. First, a comparison of biochemical criteria in the three groups showed that hCETP transgenic pigs are different from cloned and control pigs, and there were no significant differences between cloned and unmodified pigs. However, the metabolic components between cloned pigs and unmodified pigs can be distinguished by PCA analysis, indicating that the metabolic components of the two are not identical. Somatic cell nuclear transfer technology requires that single somatic cell nuclei are introduced into enucleated oocytes containing some free DNA, which may have contributed to the variation. In addition, epigenetic modifications, intestinal bacteria, and intrauterine environmental factors may have an impact on the metabolic components of cloned pigs. Studies have also shown clear differences in phenotypic or metabolic components between cloned and normal animals [30–32].

The underlying mechanism by which CETP transfers lipoprotein is still not clearly understood, and the fundamental function of CETP in nature remains unknown [9, 33]. The PLS-DA model not only distinguished the metabolic components of genetically modified pigs from unmodified pigs, but also showed discrepancy between transgenic positive and cloned pigs. The different metabolic components between hCETP transgenic and unmodified pigs might be closely associated with the physiological function of CETP, having effects on lipid metabolism, lipoprotein oxidation, inflammation, and fat synthesis [34]. Establishing the different metabolic components of hCETP transgenic positive, cloned, and unmodified pigs also needs further validation to identify the specific components, and then they can be analyzed in regards to possible mechanisms and related functions. The limitation of present study was the small sample size with only 5 pigs in each group, and a larger sample size would be more indicative of variation between animals in the multivariate analysis.

Conclusions
In conclusion, this report describes a model of lipid metabolic disturbance created by transgenesis in a large animal. The hCETP transgenic pigs showed hypercholesterolemia, and metabonomic analysis found CETP-related metabolic components. Therefore, this model should be valuable for further research into the mechanisms of CETP in the development of atherosclerosis.
