In Vivo Effects of CETP Inhibitory Peptides in Hypercholesterolemic Rabbit and Cholesteryl Ester Transfer Protein-Transgenic Mice

Kyung-Hyun Cho, Young Won Shin, Myung-Sook Choi, Song-Hae Bok, Sang-Hee Jang and Yong Bok Park*

Department of Genetic Engineering, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea
Department of Food Sciences & Nutrition, College of Human Ecology, Kyungpook National University, Taegu 702-701, Korea
Korea Research Institute of Bioscience and Biotechnology, Yusung, Taejeon 305-606, Korea
Department of Food and Nutritional Sciences, Kumi College, Kumi 730-711, Korea

Received 25 September 2001, Accepted 1 November 2001

We previously reported that cholesteryl ester transfer protein (CETP) inhibitory peptides (designated P\textsubscript{28} and P\textsubscript{10}) have anti-atherogenic effects in hypercholesterolemic rabbits (Biochim. Biophys. Acta (1998) 1391, 133-144). To further investigate those effects, we studied rabbit plasma that was collected after 30 h of a P\textsubscript{28} or P\textsubscript{10} injection. We found that there is a strong correlation between the in vivo CETP inhibition effects and alterations of lipoprotein particle size distribution in rabbit plasma, as determined on an agarose gel electrophoresis and gel filtration column chromatography. In vivo effects of the peptide were observed again in C57BL/6 mice that expressed simian CETP. The P\textsubscript{28} or P\textsubscript{10} peptide (7 µg/g of body weight) that was dissolved in saline was injected subcutaneously into the mice. The P\textsubscript{28} injection caused the partial inhibition of plasma CETP activity up to 50%, decreasing the total plasma cholesterol concentration by 30%, and increasing the ratio of HDL/total-cholesterol concentration by 150% in the CETP-transgenic (tg) mice. The CETP inhibition by the P28 or P10 made alterations that modulated the size re-distribution of the lipoproteins in the bloodstream. Particle size of the very low (VLDL) and low density lipoproteins (LDL) from the peptide-injected group was highly decreased compared to the saline-injected group (determined on the gel filtration column chromatography). In contrast, the HDL particle size of the P28-injected group increased compared to the saline-injected group (determined on the gel filtration column chromatography). These results suggest that the injection of the CETP inhibitory peptide could affect the CETP expression level in the liver by influencing lipoprotein metabolism.

Keywords: Cholesteryl ester transfer protein (CETP), Peptide inhibitor, Cholesteryl ester (CE), Lipoprotein, Cholesterol, Metabolism

Introduction

The cholesteryl ester transfer protein (CETP) plays an important role in the reverse cholesterol transport pathway which is involved in the redistribution of the lipoprotein particle size and composition (Albers et al., 1984). CETP, a 74 kDa of hydrophobic glycoprotein (Hesler et al., 1987, Jarnagin et al., 1987), promotes the transfer of cholesteryl ester (CE) from high-density lipoproteins (HDL) to apo-B that contain lipoproteins and a reciprocal transfer of triglycerides (TG) to HDL (Abbey et al., 1985). Studies with CETP-deficient patients in Japan (Koizumi et al., 1985, Inazu et al., 1990), the inhibition of CETP activity in rabbits (Abbey et al., 1989; Whitlock et al., 1989) and hamsters (Evans et al., 1994), and the introduction of human CETP into rats (Ha et al., 1985; Quig et al., 1986) and mice (Agellon et al., 1991; Hayek et al., 1992) suggest that CETP plays a major role in determining plasma lipoprotein composition, particularly the CE and TG composition of HDL.

The CETP-transgenic (tg) mouse that expressed the simian CETP gene (Marotti et al., 1992) is a valuable model for the development of a lipid-lowering drug since their lipid metabolism is very similar to that of humans. Since the mouse had a high level of plasma CETP activity with an increased LDL particle size compared to a normal C57BL/6 mouse, it promptly absorbed dietary cholesterol (Marotti et al., 1993).

The CETP inhibitory peptide was isolated from the hog plasma by ultracentrifugation, sequential column chromatographies (including Sephadex G-200, DEAE-Sephadex A-25 columns), and finally electroelution from gels (Cho et al., 1998). A peptide that was synthesized chemically, according to the amino acid sequence of the peptide
(designated P28 and P10), showed approximately the same degree of CETP inhibitory activity as the isolated peptide. The sequence of the P28 was as follows: N-Glu-Asp-Thr-Ser-Pro-Glu-Asp-Lys-Met-Gln-Asp-Tyr-Val-Lys-Gln-Ala-Thr-Arg-Thr-Ala-Gln-Asp-Ala-Leu-Thr-Ser-Val-Lys-C. The P10 peptide had ten amino acids from the amino terminal of the P28. We reported that the CETP inhibition effects of the P28 and P10 in hypercholesterolemic rabbit plasma, approximately 75% CETP activity, disappeared from the plasma within 1 h after the injection. The effect lasted up to 30 h. The inhibition of CETP in vivo led to a concomitant decrease in the total plasma cholesterol level up to 30%, and an increase in the level of HDL-cholesterol up to 32% (Cho et al., 1998).

In this report, we investigated the further inhibitory effects at the point of the lipoprotein size distribution in the rabbit plasma. We also examined the in vivo effects of the CETP inhibitory peptides using CETP-transgenic mice at the point of the CETP inhibition, as well as its influential change of lipoprotein elution profile on the gel filtration column chromatography.

### Materials and Methods

**Animals** Male CETP-transgenic C57BL6 mice-originally from Upjohn Lab (Marotti et al., 1993) (Kalamazoo, USA), 10 weeks post-weaning and weighing 25-30 g were maintained in colony cages (7 mice/cage) on 12 h-light/dark cycles. After 1 week of lab-chow feeding, the mice were allowed free access to water and a powdered semi-synthetic diet that contained high cholesterol and high fat (HCHF) for 12 weeks. The diet contained dairy butter (15%), sucrose (50%), casein (20%), cholesterol (2%), and corn oil (5%). It also contained cellulose, vitamins, and minerals (Nishina et al., 1990). After feeding the HCHF diet, the mice were randomly divided into experimental and control groups (Table 1). For the mice of the experimental groups, the P28 or P10 peptides (200 mg/200 ml, approximately final conc: 6 µM in body weight) were injected subcutaneously. Saline was injected in the control groups. 200 ml of saline was injected in the control groups. The injection was carried out twice at 0 and 12 h after the initial injection. The effect lasted up to 30 h. The inhibition of CETP activity, disappeared from the plasma within 1 h after the injection. The injection was carried out twice at 0 and 12 h after the initial injection.

**Sample collection and analyses** Blood samples were collected from the periorbital sinus into heparinized microhematocrit capillary tubes (Sigma 02-668-66) from the non-fasted male mice at designated intervals (12 h intervals). Plasma was obtained by low-speed centrifugation (5,000 g) at 4°C for 5 min and stored at −20°C until analyzed. The collected mice plasma were subjected to a CE transfer assay and cholesterol concentration determination.

To determine the size distribution of lipoproteins, the mouse plasma, which had been bled 24 h post-injection, was pooled and accessed according to the method described previously (Chen et al., 1982). One-milliliter of the pooled plasma was labeled with 1 µCi of [10,2α-(α)-3H]-cholesterol (specific activity = 50 Ci/mmol, Amersham, U.K.) by incubation at 37°C for 24 h in order to allow the free cholesterol to diffuse into all of the lipoprotein particles. The [3H]-cholesterol labeled plasma was loaded onto a Bio-Gel A-5 m column (1.5×90 cm), which had been previously equilibrated with tris-buffered saline (TBS: 10 mM Tris-Cl/140 mM NaCl/5 mM EDTA, pH 7.4). After the column application, the sample was eluted with the TBS buffer at a flow rate of 10 ml/h on gravity. The fractions were collected in 1 ml per tubes. Each 100 µl aliquot of all of the column fractions was subjected to scintillation counting using a liquid scintillation analyzer (Packard Tri Carb 1600TR) in order to visualize the eluted lipoprotein peak.

**Northern blot hybridization** To compare the expression level of CETP between the peptide and saline-injected groups, the RNA sample was isolated from the mouse liver, according to the method described by Sambrook et al. (Sambrook et al., 1989). Denatured total RNA was (20 µg) electrophoresed on 1% agarose gel that contained formaldehyde and blotted onto a Nytran-Plus membrane (Schleicher & Schull, Dassel, Germany) according to the standard protocol (Sambrook et al., 1989). The membrane was pre-hybridized and hybridized in 50% formamide, 5×SSPE (sodium chloride/sodium phosphate/EDTA), 5× Denhardt solution, 1% SDS, and 100 µg/ml denatured salmon sperm DNA at 42°C. The CETP cDNA was labeled with [α-32P]-dCTP using the Prime-a-Gene labeling system (Promega, Madison, USA). The boiled and isotope labeled CETP cDNA probe was added to the hybridization buffer for an additional 16 h. To exclude nonspecific binding, the membrane was washed at 42°C with 2× SSC (sodium chloride/sodium citrate)0.1% SDS (sodium dodecyl sulfate) for 1 h, and again at 65°C with 0.2× SSC/0.1% SDS. After the washings, the membrane was exposed to Curix RPI film (Agfa, Germany) in order to visualize the band signal.

**Lipoprotein isolation and analysis with rabbit plasma** Lipoproteins were isolated from the rabbit plasma according to the method of Sambrook et al. (Sambrook et al., 1989).

### Table 1. Experimental design of in vivo test with P28 and P10 in CETP-transgenic mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Injected material</th>
<th>Mice*</th>
<th>Initial cholesterol conc (mg/dl)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>HCHF**</td>
<td>P28 (200 µg)</td>
<td>CETP-tg</td>
<td>118.8 ± 3.5</td>
<td>7</td>
</tr>
<tr>
<td>B</td>
<td>HCHF</td>
<td>P10 (200 µg)</td>
<td>CETP-tg</td>
<td>113.0 ± 5.2</td>
<td>7</td>
</tr>
<tr>
<td>C</td>
<td>HCHF</td>
<td>Saline</td>
<td>CETP-tg</td>
<td>83.8 ± 3.5</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>Lab-chow</td>
<td>Saline</td>
<td>C57BL/6</td>
<td>41.8 ± 6.5</td>
<td>7</td>
</tr>
</tbody>
</table>

*The mice were CETP-transgenic (CETP-tg) mice that expressed cynomolgus monkey CETP (Marotti et al., 1992).

**HCHF** was high cholesterol, high fatty acid. The diet was a semi-synthetic diet which contained dairy butter (15%), sucrose (50%), casein (20%), cholesterol (2%), and corn oil (5%), as well as cellulose, vitamins, and minerals (Nishina et al., 1990).
standard method described by Havel et al. (Havel et al., 1955) with a slight modification. Briefly, pooled rabbit plasmas were brought to density 1.225 g/ml with dried solid KBr and centrifuged for 48 h at 54,000 rpm using the Ti 70.1 rotor of Beckman XL-70 centrifuge (Beckman Inc., Palo Alto, USA). After the ultracentrifugation, the lipoproteins were floated at the top of the ultracentrifuge tubes and collected. The collected lipoproteins were subjected to a Bio-Gel A-5 m column (1.5 × 90 cm) chromatography to separate the VLDL, LDL, and HDL according to their particle size (Chen et al., 1982). Electrophoretic mobility of intact rabbit lipoproteins were examined by electrophoresis in agarose gel, as described by Noble (Noble, 1968). After gel fixation and drying, the gel strip was stained for 2 h at room temperature in 50% methanol with 0.125% Coomassie brilliant blue R-250.

Other methods The cholesteryl ester transfer activity of plasma was determined as previously described (Park et al., 1992; Cho et al., 1998) using artificial substrates. Normal mouse plasma was used for determination of the non-specific CE transfer in this assay system since minimum CETP activity is reported to be present in the mouse plasma (Ha et al., 1982, Jiang et al., 1991).

Total serum cholesterol and HDL-cholesterol concentration were determined according to Allain et al. (Allain et al., 1974) using a Sigma diagnostic kit (Sigma 352-20 and 352-3, respectively).

Concentration of the protein was determined using a bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL), as described by Smith et al. (Smith et al., 1985), with bovine serum albumin as a standard.

Results and Discussion

Changes of lipoprotein size distribution in the rabbit plasma by the peptide injection Lipoproteins of the pooled rabbit plasma from each group were isolated by ultracentrifugation and Bio-Gel A-5 m column chromatography. The Bio-Gel A-5m column elution profile indicated that the VLDL and LDL protein peaks of the P28 and P10-injected groups were smaller than those of the saline-injected group (Fig. 1); whereas the HDL protein peak of the P28 and P10-injected groups were larger than those of the saline-injected group. When electrophoretic mobility of the lipoprotein fractions was measured using agarose electrophoresis, the VLDL and LDL (2 µg of protein per each lane) of the P28 and P10-injected groups migrated farther than those of the saline-injected group; whereas the HDL (6 µg of protein per each lane) of the P28 and P10-injected groups showed lower electromobility than those of the saline-injected group (Fig. 2). These results indicate that the reduced CETP activity by the P28 and P10 might cause concomitant change in size distribution of the lipoproteins in both hypercholesterolemic and normocholesterolemic rabbits.

Changes of CETP activity in CETP-transgenic mice When the peptides (P28 or P10) were subcutaneous injected into the mice, the CETP activity of group A (HCHF-fed, P28-injected) decreased by 50% compared to the initial activity at 24 h post-injection. The CETP activity of group B (HCHF-fed, P10-injected) decreased by 23% compared to the initial activity at 36 h post-injection; whereas the activity of group C (HCHF-fed, saline injected, CETP-tg mice) increased by 53% compared to the initial activity at 36 h post-injection (Fig. 3). The CE-transfer activity of group C showed a slight increasing tendency with progress of bleeding time. Since normal mice do not have CETP activity (Ha et al., 1982, Jiang et al., 1991), the CETP activity of group D (chow-fed, saline-injected, normal mice) was not determined.

Changes of total serum cholesterol level and HDL-cholesterol ratio After feeding a semi-synthetic diet that contained 2% cholesterol for 12 weeks, the CETP-tg mice
In vivo Effects of CETP Inhibitory Peptide 175 became hypercholesterolemic mice. They have 80-120 mg/dl of total serum cholesterol, in contrast to 40-50 mg/dl before the HCHF feeding.

The total cholesterol concentration of group A (P28-injected) decreased by 30% compared to its initial concentration at 24 h post-injection. Group B (P10-injected) decreased by 18% compared to the initial value at 36 h post-injection. The total cholesterol concentration of group C (saline-injected) increased by 116% compared to its initial value. Group D (saline-injected) showed no notable change in total cholesterol concentration (Fig. 4, panel A). The ratio of the HDL-cholesterol/total-cholesterol (HDL-C/Total-C) of Group A increased by 170% and 250% in group B compared to its initial value 24 h after the initial injection. The ratio of HDL-C/Total-C of Group C decreased by 17% to the initial value. Group D showed no significant change (panel B of Fig. 4). These results indicate that the blocking of the CETP activity in plasma could cause decreased total cholesterol and increased HDL-cholesterol concentration.

Changes of lipoprotein size distribution in the blood of mice As shown in panels C and D of Fig. 5, the Bio-Gel A-5m column elution profile of mice plasma showed typical patterns of hypercholesterolemic CETP-tg mouse and normal C57BL/6 mice, respectively, as previously reported (Marotti et al., 1993). However, the CETP inhibition by the P28 or P10 might cause modulation of size distribution of LDL and HDL in the bloodstream. As shown in Fig. 5, 24 h post-injection, VLDL and LDL peak size of the peptide-injected group (panel A, B) were smaller than the saline-injected group (panel D). In contrast, the peak size of HDL from the peptide-injected group (panels A, B) was larger than that of the saline-injected group (panel D).

Difference of CETP expressional level in liver As shown in Fig. 6, the CETP band did not appear in normal C57BL/6 mice (lane 4), as previously reported (Ha et al., 1982, Jiang et al., 1991), but the saline-injected CETP-tg mouse represented a high expression level of CETP (lane 3), as previously reported by Marotti et al. (1993). The CETP mRNA expression level of the P28-injected CETP-tg mouse (lane 1)
was lower than that of the saline-injected CETP-tg mouse. This result suggests that the P28 might affect the CETP expression level in the liver of the hypercholesterolemic CETP-tg mouse.

It can be concluded that the P28 or P10 injection might cause partial inhibition of plasma CETP activity by decreasing the total plasma cholesterol concentration and increasing the ratio of the HDL/total-cholesterol concentration in hypercholesterolemic CETP-tg mice. These results agree with the in vivo observations, which revealed clear relationships between plasma CE transfer activity and size distribution of both HDL and LDL. (Agellon et al., 1991; Lagrost, 1993).

Acknowledgment This research was supported by a grant (#1999-2-220-005-5) from the Korea Science and Engineering Foundation (KOSEF).

Fig. 4. Effect of peptide on total cholesterol (A) and HDL-C/Total-C ratio (B) in hypercholesterolemic CETP-transgenic mice. All of the mice were fasted for 16 h before injection. Arrows indicate the time of injection. The mice in each group (n=7) were bled, and HDL-C and Total-C determined using Sigma diagnostic kits (Sigma 352-3 and 352-20, respectively). Every data are expressed as a percent of the initial value. HCHF was the abbreviation of the high cholesterol high fat-diet described in Table 10. Asterisk symbols (*) on the x-axis indicate a statistically significant difference between the saline-injected and at least one of peptide-injected groups at the corresponding time point by a one-way analysis of the variance and Duncan’s multiple range test (p<0.05).

Fig. 5. Elution profile of Bio-Gel A-5 m column chromatography (1.5×90 cm) with tritiated mouse plasma. The plasma was radiolabeled with 1 µCi of [1α,2α-(n)-3H]-cholesterol at 37°C for 24 h as previously described (Chen et al., 1982). The column was equilibrated with 10 mM Tris/140 mM NaCl that contained 1 mM EDTA, pH 7.4, (Tris buffered saline, TBS) eluted with the same buffer. One-ml fraction was collected with a flow rate of 10 ml/hr. An aliquot (100 µl) of the column fractions was subjected to liquid scintillation counting to visualize lipoprotein peak. Panel A, the P28-injected, high cholesterol high fat (HCHF)-fed; panel B, P10-injected, HCHF-fed; panel C, saline-injected, HCHF-fed; panel D, saline-injected, Lab-chow-fed.

Fig. 6. Northern blot analysis of the CETP gene. Total RNA (20 µg) of each group from the liver was loaded on each lane and hybridized with α-[32P]-labeled CETP cDNA as detailed in “Materials and Methods”. Control hybridization was done with a probe for the GAPDH gene after washing the blot in 0.1% SDS at 65°C. Lane 1, group A (P28-injected, CETP-tg mice); lane 2, group B (P10-injected, CETP-tg mice); lane 3, group C (saline-injected, CETP-tg mice); lane 4, group D (saline-injected, C57BL/6 normal mice).

References


In vivo Effects of CETP Inhibitory Peptide


