Changes in Plasma Lipoprotein Cholesterol Levels by Antisense Oligodeoxynucleotides against Cholesteryl Ester Transfer Protein in Cholesterol-fed Rabbits*

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Cholesteryl ester transfer protein (CETP)1 is the enzyme that facilitates the transfer of cholesteryl ester from high density lipoprotein (HDL) to apoB-containing lipoproteins and also affects the low density lipoprotein metabolism. On the other hand, the liver is the major tissue responsible for the production of CETP mRNA in rabbits. To test the hypothesis that a reduction of CETP mRNA in the liver by antisense oligodeoxynucleotides (ODNs) may affect the plasma lipoprotein cholesterol levels, we intravenously injected antisense ODNs against rabbit CETP coupled with asialoglycoprotein carrier molecules, which serve as an important method to regulate liver gene expression, to cholesterol-fed rabbits via their ear veins. All rabbits were fed a standard rabbit chow supplement with 0.1% cholesterol for 10 weeks before and throughout the experiment. After injecting rabbits with antisense ODNs, the plasma total cholesterol concentrations and plasma CETP activity decreased at 24, 48, and 96 h, whereas the plasma HDL cholesterol concentrations increased at 48 h. A reduction in the hepatic CETP mRNA was also observed at 6, 24, and 48 h after the injection with antisense ODNs. However, in the rabbits injected with sense ODNs, the plasma total and HDL cholesterol concentrations and the plasma CETP activities did not significantly change, and the hepatic CETP mRNA did not change either throughout the experimental period. Although the exact role of CETP in the development of atherosclerosis remains to be clarified, these findings showed for the first time that the intravenous injection with antisense ODNs against CETP coupled to asialoglycoprotein carrier molecules targeted to the liver could thus inhibit plasma CETP activity and, as a result, could induce a decrease in the plasma low density lipoprotein and very low density lipoprotein cholesterol and an increase in the plasma HDL cholesterol in cholesterol-fed rabbits. (3). The homozygotes for CETP deficiency demonstrated markedly elevated HDL-C and plasma apoA-I levels as well as decreased LDL cholesterol and plasma apoB levels (4, 6). CETP-deficient subjects have also been found to have a substantially increased catabolic rate of apoB as the primary metabolic basis for the low plasma levels of LDL apo B (7). This finding indicates that the LDL receptor pathway may thus be up-regulated during CETP deficiency. It has also been proposed that a CETP deficiency may be associated with protection against ischemic heart disease, based on the observed longevity in one kindred (3), as well as the lack of any evidence of coronary heart disease (6) in other kindreds with CETP deficiency; however, these findings remain controversial. Several other lines of evidence also support the hypothesis. The plasma level of CETP is directly correlated with the extent of coronary atherosclerosis in monkeys fed a cholesterol diet (8). A transgenic mouse overexpressing simian CETP developed accelerated atherosclerosis (9). Thus, the inhibition of plasma CETP activity may potentially be a novel method of reducing the plasma levels of LDL cholesterol by enhancing LDL catabolism (7) and decreasing the transfer of cholesteryl ester from HDL to apoB-containing lipoproteins (1, 2). Since the liver is the major tissue responsible for the production of CETP (CETP mRNA) in rabbits (10, 11) (even though adipose tissue may also be the major tissue responsible for the production of CETP in monkeys (12)), a reduction of CETP in the liver by antisense oligodeoxynucleotides (ODNs) may thus cause a reduction in the plasma LDL and/or VLDL cholesterol concentrations. The present study was therefore undertaken to determine the effect of an intravenous injection with antisense ODNs to the liver on the CETP mRNA expression, plasma CETP activity and plasma cholesterol concentrations in rabbits fed a low cholesterol diet. These antisense ODNs were originally designed to be coupled with asialoglycoprotein carrier molecules, and this coupling serves as an important method to regulate liver gene expression (13).

MATERIALS AND METHODS

Construction of ODNs—The sequences of ODNs against rabbit CETP used in this study were as follows: antisense, 5'-CTTGACCCGGC-CGAGGAGCAT-3'; sense, 5'-ATGCCCTCGGCCTGAAGCAAG-3'; positions +148 to +168 of the rabbit sequence (11). These selected target sequences have relatively low homology with any of the other known cDNA sequences found in the GenBank database. The synthetic ODNs were purified on the column, dried down, resuspended in Tris-EDTA (10 mM Tris, pH 7.4, and 1 mM EDTA), and then quantitated by spectrophotometry. Asialoglycoprotein-poly-l-lysine (M, approximately 71,400), which was prepared according to the method of Wu and Wu (14) and Wu et al. (15), was added to the ODNs (at a molar ratio of 25:1 with vigorous mixing. The solution was incubated at 4 °C overnight and dialyzed (twice) against 0.15 M saline (1500:1; membrane M, cutoff, 3500). The samples were electrophoresed through a 2% agarose gel using Tris/borate/EDTA buffer and then stained with ethidium bromide to visualize DNA. The samples were filtered through a 0.2-μm mem-

1 The abbreviations used are: CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; LDL, low density lipoprotein; ODN, oligodeoxynucleotide; VLDL, very low density lipoprotein; PCR, polymerase chain reaction.
bran (Millipore Corp., Bedford, MA) before injection.

Experimental Protocol—Twenty-six male Japanese white rabbits weighing 2.0–2.5 kg were used in the experiment. All animals were housed individually, had free access to water, and were fed a standard rabbit chow supplement with 0.1% cholesterol for 10 weeks before and throughout the experiment. The plasma total and HDL cholesterol concentrations, which did not significantly change between the period after 9 and 10 weeks of feeding, were determined. Thirteen animals were injected with asialoglycoprotein-poly-L-lysine-sense ODN complex, whereas the remaining 13 animals were injected with asialoglycoprotein-poly-L-lysine-antisense ODN complex via the ear veins. The amount of ODNs injected was 30 μg/kg for each rabbit. At 6, 24, 48, and 96 h after injection, two rabbits in each group were killed, and liver specimens were taken. At the same time, about 1 ml of the blood was drawn from the remaining animals via their ear veins.

Measurement of CETP mRNA—Total RNA was isolated from the liver with a RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s procedure with slight modifications (12). The abundance of CETP mRNA was determined by quantitative dot blotting (16). The rabbit cRNA probe labeled with fluorescein-dUTP was produced from the plasmid containing the CETP cDNA using the nonradioactive, reverse transcription polymerase chain reaction (PCR) (Amersham Corp.), according to the rabbit sequence (11). The sense and antisense primers used for PCR, the sizes of the PCR products, and the PCR cycles in each cRNA probe were: CETP, sense, 5'-CTTTCATTAACCTGCTCTG-3'; antisense, 5'-CTTGGGCTTCCGGCACTTTTCT-3'; size, 482 base pairs; 30 cycles; and glyceroldehyde-3-phosphate dehydrogenase, sense, 5'-ATGGTCTACATGTTCTGTC-3'; antisense, 5'-AAAGCAATTGGGTTGCGAGC-3'; size, 343 base pairs; 30 cycles.

Biochemical Analysis—The plasma cholesterol concentrations were measured in whole plasma and in the HDL-containing supernatant after the precipitation of VLDL and LDL with dextran-Mg2+ using the Wako total and HDL cholesterol measuring kit (Wako Ltd., Osaka, Japan). The plasma constituents related to liver function were analyzed using an automatic analyzer (Hitachi Ltd., Tokyo, Japan). The CETP activity in the plasma was determined by a radioassay according to the modified method of Yen et al. (17). A volume of 20 μl of plasma was incubated for 30 min at 37°C in the presence of [14C]cholesteryl oleate-d3-labeled LDL (3–10 nmol CE) and an excessive amount of VLDL and LDL (0.2 μmol of CE). The volume was adjusted to 200 μl with Tris-saline (pH 7.4) before incubation. After the precipitation of VLDL and LDL by heparin and MnCl2 (18), half of the supernatant volume was then removed and counted in a liquid scintillation counter.

Statistical Analysis—All values presented are the mean ± standard error of the mean. The statistical analysis was performed by a paired t test for comparisons in the intragroup and by Student’s t test for comparisons between the groups. Differences were considered statistically significant at a value of p < 0.05.

RESULTS

We characterized the asialoglycoprotein-ODN complex by gel electrophoresis. The samples were electrophoresed through a 2% agarose gel using Tris/borate/EDTA buffer and then were stained with ethidium bromide to visualize DNA (Fig. 1). The ODNs were retained by the asialoglycoprotein-poly-L-lysine conjugate in the well, whereas ODNs alone entered the gel. In the rabbits injected with antisense ODNs, the total cholesterol concentrations and the CETP activities were all significantly decreased at 24, 48, and 96 h compared with those at 0 h. At 48 h, the total cholesterol concentrations and the CETP activities were also significantly lower in the rabbits injected with antisense ODNs than in those injected with sense ODNs (Fig. 2). The HDL cholesterol concentrations significantly increased at 48 h compared with those at 0 h and the rabbits injected with sense ODNs (Fig. 2). In the rabbits injected with sense ODNs, the total and HDL cholesterol concentrations and the CETP activities did not significantly change throughout the experiment (Fig. 2). Fig. 3 shows a typical example of the dot blot analyses of hepatic CETP mRNA treated with antisense ODNs. A reduction of hepatic CETP mRNA was observed at 6, 24, and 48 h after injection with antisense ODNs. When the amount of hepatic CETP mRNA was measured by scanning and expressed as a ratio to glyceraldehyde-3-phosphate dehydrogenase mRNA, the mean values were 0.83 (100%) at 0 h, 0.43 (51.8%) at 6 h, 0.40 (48.2%) at 24 h, 0.65 (78.3%) at 48 h, and 0.87 (104.8%) at 96 h (the parentheses express the percentages against the value at 0 h). Hepatic CETP mRNA treated with sense ODNs did not change throughout the experimental period (data not shown). We measured the plasma constituents related to liver function (aspartate aminotransferase, alanine aminotransferase γ-GTP, alkaline phosphatase, and total bilirubin), including triglyceride in the rabbits (data not shown). These levels did not significantly change throughout the exper-
always show the true CE mass transfer

The assay used for the CETP activity in this study cannot
these results, the following factors are considered to play a role. The assay used for the CETP activity in this study cannot
and the LDL receptor is down-regulated, and CETP mRNA in
and plasma CETP increase especially in the rabbits fed an atherogenic diet more than in those fed a standard diet
also increase the increase in the LDL receptor much more than other models. Thus, as a result, the VLDL and LDL cholesterol levels might be reduced more than the HDL level was increased. Our anti-
sense injection was considered successful for the following reasons: (a) the asialoglycoprotein-poly-l-lysine-antisense complex is rapidly and preferentially taken up by the liver (13) and has enhanced resistance to nuclease degradation in plasma (31); (b) the amount of CETP mRNA in the liver is thought to be relatively low compared with other lipoprotein mRNAs in the liver; however, these findings have only been previously seen in the cynomolgus monkey (12); and (c) the liver is the major tissue responsible for the production of CETP (CETP mRNA) in rabbits (10, 11) (although adipose tissue may also be found in monkeys (12)). The exact role of CETP in the development of atherosclerosis has yet to be clarified. Marotti et al. (9) demonstrated that transgenic mice expressing cynomolgus monkey CETP had significantly more early atherosclerotic lesions in the proximal aorta than controls when fed a high cholesterol diet. On the other hand, more recently Hayek et al. (32) concluded that CETP expression inhibited the development of early atherosclerotic lesions in hypertriglyceridemic mice. The CETP expression in hypertriglyceridemic animals produced a much greater reduction in the HDL size (33). These small particles, which can be produced by CETP (34), may thus be an optimal mediator of cellular cholesterol efflux (35).

In conclusion, in this study we have shown that the intravenous administration of the asialoglycoprotein-poly-l-lysine-antisense complex is a beneficial method for reducing the plasma levels of LDL and VLDL cholesterol and increasing the plasma level of HDL cholesterol, possibly by enhancing LDL catabolism (7) and decreasing the transfer of cholesteryl ester from HDL to apoB-containing lipoproteins (1, 2). However, it must be mentioned that our results were limited to the period comprising only several days after the injection. Therefore, to elucidate the exact effect of CETP on atherosclerosis development, further longer term studies are called for.

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