Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 519-524

CHOLESTERYL ESTER TRANSFER PROTEIN DEFICIENCY CAUSED BY A NONSENSE MUTATION DETECTED IN THE PATIENT'S MACROPHAGE mRNA

Takanari Gotoda*, Makoto Kinoshita§, Hitoshi Shimano, Kenji Harada, Masako Shimada, Jun-ichi Ohsuga, Tamio Teramoto§, Yoshio Yazaki, and Nobuhiro Yamada

The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

§The First Department of Internal Medicine, Teikyo University School of Medicine, Tokyo 173, Japan

Received June 8, 1993

Cholesteryl ester transfer protein (CETP) deficiency causes marked elevation of plasma high-density lipoproteins, termed hyperalphalipoproteinemia. Only one CETP mutation has been found previously, partly because the relative unavailability of CETP mRNA has hampered analysis. We demonstrated CETP mRNA expression in the monocyte-derived macrophages and identified a new CETP mutation by analyzing the macrophage mRNA of a homozygous patient with familial form of hyperalphalipoproteinemia. The nonsense mutation at codon 309 in exon 10 of the CETP gene was thought to delete the carboxy-terminal third and caused a decrease in the level of CETP mRNA. Our findings provide more evidence that CETP mutations may underlie a subset of familial hyperalphalipoproteinemia.

Hyperalphalipoproteinemia, a condition associated with an increase of plasma high-density lipoprotein (HDL) cholesterol, is a negative risk factor for coronary heart disease and sometimes clusters in families (1). Plasma cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl esters from HDL to other lipoproteins and regulates plasma HDL levels (2-4). Thus, mutations of the CETP gene are a likely cause of familial hyperalphalipoproteinemia, which is transmitted as an autosomal dominant trait (1). Previously, an intron 14 donor splice site mutation was found in the CETP gene of some Japanese patients with familial hyperalphalipoproteinemia (5-7). However, no other CETP gene abnormalities have been discovered since, casting some doubt on the generality of

* To whom correspondence should be addressed.

Abbreviations : CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; PBS, phosphate-buffered saline; RT-PCR, reverse-transcription polymerase chain reaction; CE, cholesteryl ester.

0006-291X/93 $4.00

the relationship between CETP mutations and familial hyperalphalipoproteinemia. So far, molecular analysis of the CETP gene has focused on extensive sequencing of 16 exons, because tissue biopsy has been required for the analysis of CETP mRNA.

Here we report the identification of a novel CETP mutation in a patient with familial hyperalphalipoproteinemia by employing monocyte-derived macrophages as the source of CETP mRNA.

MATERIALS AND METHODS

Proband : The proband was a 45-year-old male member of a Japanese family with a cluster of hyperalphalipoproteinemic individuals. His parents were first cousins. He had no clinical signs of atherosclerosis or xanthomatous accumulation and there was no significant history of cardiovascular disease in the family. As reported in some other families with familial hyperalphalipoproteinemia (7), several members have had an increased life span. His fasting plasma total cholesterol and triglyceride levels were 256 and 65 mg/dl, respectively, whereas the HDL-cholesterol level was 179 mg/dl (nearly four times the normal) and the apolipoprotein A-I level was 222 mg/dl. The levels of his plasma CETP activity and mass, which were measured according to the methods reported previously (8,9), were both less than 5% of the control. Thus, the phenotype of the proband was both clinically and biochemically indistinguishable from those of other patients homozygous for the known splicing mutation (5-7).

Preparation of Monocyte-derived Macrophages : Monocyte-derived macrophages were prepared by culturing human monocytes. Mononuclear cells were isolated from peripheral blood cells by the Ficoll-Hypaque gradient method as described previously (10). The cells were washed three times with phosphate-buffered saline (PBS). The washed cells were suspended in RPMI-1640 and plated in 10-cm dishes (2×10^7 cells/dish). After two hours of incubation at 37°C in 5% CO_2 and 95% air, non-adherent cells were removed by three washes with PBS. The cells were placed again in fresh RPMI-1640 medium supplemented with 10% autologous serum and then cultured for ten days. The medium was changed twice.

Amplification of mRNA : The CETP mRNA was studied by the reverse-transcription polymerase chain reaction (RT-PCR), as described previously (10). The oligonucleotide primers used were the following: F1=5'-GGGCCACTTACACACCA C-3', F2=5'-ACACCACTGCCTGATAACC-3', F3=5'-ACCTGGAGTCCCATCACAAG-3', F4=5'-GCTTCAACACCAACCAGGAA-3', R1=5'-AACCAGAAGTACAGCATGCG-3', R2=5'-GTGCTTGCCTTCTGCTACAA-3', R3=5'-ACACCAGGGTTCCAGCTGTGA -3', R4=5'-TTGCTGGTCTGGGCGTGG-3', R5=5'-GCAGGATTGGGGTACGTGA-3'. The primers were synthesized according to the published sequence data (11,12). After reverse transcription and the first PCR with primers F1 and R3, the DNA product containing nucleotides 101-1685 of human CETP cDNA (11) was subjected to a second PCR with primers F2 and R1 to amplify fragment A (nucleotides 112-975) or with primers F3 and R2 to amplify fragment B (nucleotides 861-1659). These two fragments cover the entire CETP coding region.

Quantitation of mRNA by Competitive PCR : CETP mRNA levels were measured by the competitive PCR method (10,13). RNA from normal and patient macrophages was mixed in a series of ratios and then the mixtures (1 μg RNA) were reverse-transcribed with primer R3, amplified with primers F4 and R4, and digested with restriction enzyme *Dde I*. The relative amounts of the normal and abnormal products reflect the original contents of normal and patient CETP mRNA. RNA preparation and DNA sequencing were carried out as described elsewhere (10).

RESULTS AND DISCUSSION

Human CETP mRNA is expressed in the liver, small intestine, spleen, adrenal (11), adipose tissue (14), and placenta (15), but these specimens were unavailable from the present patient. Instead, we employed monocyte-derived macrophages as the source of CETP mRNA, because Faust et. al have reported that cultured monocyte-derived macrophages produced CETP (16). The RT-PCR with CETP-specific primers amplified cDNA fragments of the expected sizes from normal macrophage RNA (Fig. 1). DNA sequencing demonstrated that the amplified fragments were really from CETP mRNA, and also showed that the sequence of CETP mRNA from the macrophages was completely identical with that of CETP mRNA from the liver (11).

Direct sequencing of the amplified CETP cDNA fragments from the patient revealed a C to T change at codon 309 in exon 10 which replaced a codon for glutamine (CAA) with a premature stop codon (TAA) (Fig. 2). No other sequence alteration was found in the entire CETP coding region of the patient's cDNA.

Since the nonsense mutation created a new site for restriction enzyme *Dde I*, digestion of the amplified genomic DNA and cDNA with *Dde I* helped to confirm that the proband was truly homozygous for the mutation (Fig. 3). Also, co-amplification of RNA from normal and patient macrophages and subsequent digestion with *Dde I* showed that the CETP mRNA level in the patient was about 1/10 of that in the normal control (Fig. 4).

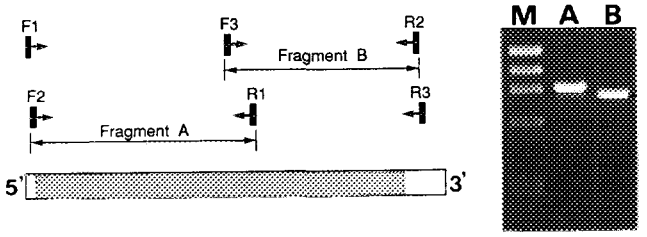

Fig. 1. *RT-PCR amplification of CETP mRNA.* (Left) Diagram of the amplified regions of human CETP cDNA. The hatched box denotes the CETP coding region and the arrows indicate the primers used for the RT-PCR. Total RNA (1 μg) isolated from normal monocyte-derived macrophages was incubated for 60 min at 37°C in reaction mixture (25 μl) containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 5 nmol of each dNTP, 5 units of ribonuclease inhibitor from human placenta (TAKARA SHUZO, Kyoto, Japan), 15 pmol of primer R3 and 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). The total products were subjected to the first PCR using an equal volume (25 μl) of solution containing 50 mM KCl, 10 mM Tris (pH 8.3), 0.01% gelatin, 5 nmol of each dNTP, 5 μl dimethyl sulfoxide, 15 pmol of primer F1 and 2.5 units of Taq DNA polymerase. PCR-amplification (25 cycles) was performed under the following condition: 1-min denaturation at 94°C, 2-min primer annealing at 50°C and 3-min extension at 72°C. To amplify the fragments A and B, 1/1000 of the first PCR products were subjected to the second PCR. These two fragments cover the entire CETP coding region. (Right) Electrophoretic analysis of RT-PCR products from normal macrophage RNA. M=molecular size marker (øx174/Hae III), A=fragment A, B=fragment B.

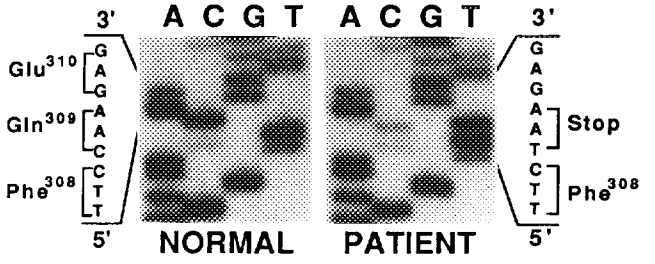

Fig. 2. *DNA sequences of CETP cDNA from a normal control and the patient.* The C to T substitution at the first nucleotide of codon 309 causes premature termination of the translation of mutant CETP mRNA.

Recently, two groups independently gave evidences that the carboxy-terminus of human CETP is essential for normal cholesteryl ester (CE) transfer activity (15, 17,18). With site-directed mutagenesis and expression study in baculovirus-transfected insect cells, Au-Young and Fielding showed that CETP mutants with carboxy-terminal deletions of 26, 48, and 66 amino acids were defective in neutral lipid transfer (CE and triglyceride transfer) as well as in lipoprotein binding. They proposed that the sequence Phe-Leu-Leu-Leu (residues 454-457 of human CETP) is, at least in part, required for both normal lipoprotein binding and catalysis of transfer activity based on results of helical wheel analysis of the amino acid sequences which are conserved between CETP and several other proteins whose functions involve binding nonpolar lipids (15). By expressing in COS cells a series of CETP mutants with small deletions or point mutations in the carboxy-terminus,

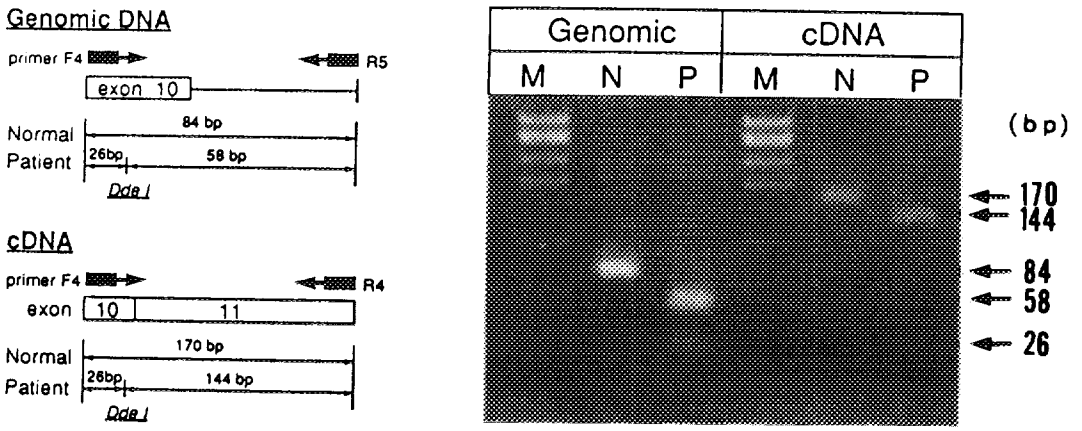

Fig. 3. *Detection of the nonsense mutation by digestion with Dde I.* (Left) Schematic representation of the amplified portion of the CETP gene (upper) and CETP cDNA (lower). The created *Dde I* site is indicated by a vertical line. The numbers show the sizes of the fragments generated upon digestion with *Dde I*. (Right) Electrophoretic analysis of the digested products on a 1.5% agarose gel. M = molecular size standard (øX174/Hae III), N = normal control, P = patient . The result established that both fragments amplified from genomic DNA and cDNA of the patient contained a new recognition site for *Dde I*.

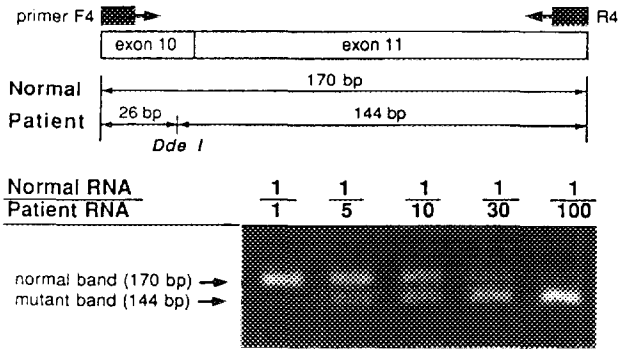

Fig. 4. *Quantitation of mRNA by the competitive PCR.* (Upper) Diagram of the amplified region. The created *Dde I* site is indicated by a vertical line. The numbers show the sizes of the fragments generated upon digestion of the RT-PCR products with *Dde I*. (Lower) RNA from normal and patient macrophages was mixed in the ratios indicated in the figure and then the mixtures (1 μg RNA) were reverse-transcribed with primer R3, amplified with primers F4 and R4, and digested with *Dde I*. The relative amounts of the normal 170-bp and abnormal 144-bp bands reflect the original contents of normal and patient CETP mRNA. The two bands were almost equally amplified when RNA from the normal subject was mixed with that from the patient at a ratio of 1:10, suggesting that the CETP mRNA level in the patient was about 1/10 of that in the normal subject.

Wang et al. also showed that deletions within amino acids between residues 463-475 selectively impair neutral lipid transfer activity but not phospholipid transfer activity (17) and that the bulky hydrophobic amino acids of a carboxy-terminal amphipathic helix of CETP (especially Leu^{475}, Phe^{471}, Leu^{468}, Phe^{461}, and Phe^{454}) is involved in neutral lipid transfer (18). Thus, the mutant CETP of the patient, which lacks the carboxy-terminal 168 amino acids (residues 309-476), could never be active in CE transfer. This finding, as well as the decreased level of CETP mRNA, provides a molecular basis for CETP deficiency in our patient.

The detection of another CETP mutation in a patient with familial hyperalphalipoproteinemia may suggest that such mutations are a relatively frequent cause of familial hyperalphalipoproteinemia in humans. Taking account of the complex structure of the CETP gene (12), it seems quite likely that many other abnormalities are still undetermined in this gene locus. Now that it has been demonstrated that CETP mRNA can be obtained from monocyte-derived macrophages, the detection of CETP mutations should be facilitated.

REFERENCES

1. Breslow, J.L. (1989) In The Metabolic Basis of Inherited Disease (C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, Eds.) 6th Ed., pp.1251-1266. McGraw-Hill, New York.
2. Morton, R.E., and Zilversmit, D.B. (1983) J. Biol. Chem. 258, 11751-11757.
3. Albers, J.J., Tollefson, J.H., Chen, C.-H., and Steinmetz, A. (1984) Arteriosclerosis 4, 49-58.
4. Tall, A.R. (1986) J. Lipid Res. 27, 361-367.

5. Brown, M.L., Inazu, A., Hesler, C.B., Agellon, L.B., Mann, C., Whitlock, M.E., Marcel, Y.L., Milne, R.W., Koizumi, J., Mabuchi, H., Takeda, R., and Tall, A.R. (1989) Nature 342, 448-451.
6. Yamashita, S., Hui, D.Y., Sprecher, D.L., Matsuzawa, Y., Sakai, N., Tarui, S., Kaplan, D., Wetterau, J.R., and Harmony, J.A.K. (1990) Biochem. Biophys. Res. Commun. 170, 1346-1351.
7. Inazu, A., Brown, M.L., Hesler, C.B., Agellon, L.B., Koizumi, J., Takata, K., Maruhama, Y., Mabuchi, H., and Tall, A.R. (1990) N. Engl. J. Med. 323, 1234-1238.
8. Kinoshita, M., Arai, H., Fukasawa, M., Watanabe, T., Tsukamoto, K., Hashimoto, Y., Inoue, K., Kurokawa, K., and Teramoto, T. (1993) J. Lipid Res. 34, 261-268.
9. Fukasawa, M., Arai, H., and Inoue, K. (1992) J. Biochem. 111, 696-698.
10. Gotoda, T., Yamada, N., Murase, T., Inaba, T., Ishibashi, S., Shimano, H., Koga, S., Yazaki, Y., Furuichi, Y., and Takaku, F. (1991) J. Biol. Chem. 266, 24757-24762.
11. Drayna, D., Jarnagin, A.S., McLean, J., Henzel, W., Kohr, W., Fielding, C., and Lawn, R. (1987) Nature 327, 632-634.
12. Agellon, L.B., Quinet, E.M., Gillette, T.G., Drayna, D.T., Brown, M.L., and Tall, A.R. (1990) Biochemistry 29, 1372-1376.
13. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H.F. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2725-2729.
14. Jiang, X.C., Moulin, P., Quinet, E., Goldberg, I.J., Yacoub, L.K., Agellon, L.B., Compton, D., Schnitzer-Polokoff, R., and Tall, A.R. (1991) J. Biol. Chem. 266, 4631-4639.
15. Au-Young, J., and Fielding, C.J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4094-4098.
16. Faust, R.A., Tollefson, J.H., Chait, A., and Albers, J.J. (1990) Biochim. Biophys. Acta. 1042, 404-409.
17. Wang, S., Deng, L., Milne, R.W., and Tall, A.R. (1992) J. Biol. Chem. 267, 17487-17490.
18. Wang, S., Wang, X., Deng, L., Rassart, E., Milne, R.W., and Tall, A.R. (1993) J. Biol. Chem. 268, 1955-1959.