Apolipoprotein E Polymorphism Determined by Restriction Enzyme Analysis of DNA Amplified by Polymerase Chain Reaction: Convenient Alternative to Phenotyping by Isoelectric Focusing

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Three common alleles determine six apolipoprotein E (apo E) phenotypes that are associated with variations in serum cholesterol in the population. This genetic variation results from single nucleotide alterations at two DNA loci encoding the amino acid residues 112 and 158 of apo E. We compared results of apo E phenotyping carried out by isoelectric focusing with those of apo E genotyping accomplished by direct DNA analysis. In the latter, the target DNA was amplified by the polymerase chain reaction (PCR) and subsequently analyzed by digestion with the restriction enzyme Hha I, followed by polyacrylamide gel electrophoresis of the cleavage products. With one exception, these two techniques yielded similar results from all 40 samples tested. In addition, a rare variant form of apo E (phenotype E1) was analyzed separately and incorrectly diagnosed as E2 by the Hha I digestion method; the anticipated mutation in the codon 127 was, however, confirmed by demonstration of a new Taq I restriction site in this variant gene. These data confirm that the common isoforms of apo E usually arise from genetic variation of the codons 112 and 158 and demonstrate the feasibility of the PCR technique in apo E genotyping.

Additional Keyphrases: hyperlipoproteinemia · cholesterol · risk factors

Apolipoprotein E (apo E) is an important structural constituent of several serum lipoprotein classes, including VLDL (very-low-density lipoprotein), chylomicrons, and HDL (high-density lipoprotein).6 It also serves as the ligand for LDL (low-density lipoprotein) and chylomicron-receptormembran receptors (1–4). A recently described LDL-receptor-related protein, the function of which is still unknown, also appears to use apo E as a ligand (5). Aside from functioning in lipoprotein metabolism, apo E may be involved in the repair responses to tissue injury as well as in immunoregulation and modulation of cell growth (6). Apo E is synthesized in most organs and cells of the body, especially in liver, brain, spleen, kidney, and macrophages (4). The apo E structural gene, containing four exons and three introns, resides in chromosome 19 and codes for a mature apo E polypeptide of 299 amino acids (6–8).

Three common isoforms of apo E (E2, E3, and E4), encoded by three different alleles (e2, e3, and e4), exist in the population (2, 4). In different combinations, these three alleles determine six different phenotypes of apo E. This genetic variation, the result of cysteine–arginine interchanges at two locations (residues 112 and 158) of the amino acid sequence of apo E (9), has been demonstrated to result from single nucleotide changes in the fourth exon of the apo E gene (8, 10). Approximately 90% of subjects with type III hyperlipoproteinemia (primary dysbetalipoproteinemia), a disease characterized by xanthomatosis, premature atherosclerosis, and high concentrations of triglyceride and cholesterol in serum, are homozygous for the e2 allele, which encodes for cysteine instead of the normal arginine at residue 158 (2, 4, 11, 12). Hyperlipidemia in this disorder most likely results from defective binding of apo E2-containing remnant lipoproteins to hepatic lipoprotein receptors (13). However, only ~1%–2% of people homozygous for the e2 allele develop type III hyperlipproteinemia, suggesting that other genes or environmental factors are necessary for disease expression (5). In the phenotype E4, arginine is substituted for cysteine at residue 112 of apo E. The presence of apo E4 is associated with increased concentrations of LDL cholesterol in serum (14–17). Gregg et al. (16) suggested that rapid catabolism of apo E4-containing lipoprotein remnant particles, with resulting down-regulation of hepatic LDL receptors, may lead to increased concentrations of LDL cholesterol in serum. As an alternative hypothesis, Kesäniemi et al. (18) maintain that the intestinal cholesterol absorption efficiency is related to apo E phenotypes, with absorption values being greatest in those with phenotypes E4/3 and E4/4. Whatever the mechanism, as much as 8% of the total variation of the concentration of LDL cholesterol in serum may be accounted for by the apo E gene locus (19). The importance of the genetic variation of this gene locus is further substantiated by findings showing an association between the apo E4 phenotype and increased risk of myocardial infarction (20–24).

Determination of apo E phenotypes may be accomplished by two-dimensional gel electrophoresis (25) or isoelectric focusing (26). The fact that the two variable nucleotides are located only 138 basepairs (bp) from each other renders the polymorphic area of the apo E gene an especially favorable target for amplification with the polymerase chain reaction (PCR), followed by hybridization analysis with allele-specific oligonucleotide probes (27–32). Here we describe the validation of a convenient PCR technique for determining the two variable nucleotides at codons 112 and 158 of the apo E gene. In addition to DNA amplification by PCR, we take advantage of the fact that nucleotide substitutions at these two loci generate restriction fragment length polymorphisms that can be detected with the enzyme Hha I (33). Applying the same principle, we also demonstrate that a rare variant form of apo E, showing a phenotype E1 by isoelectric focusing (34), results from a single base substitution at codon 127, which generates a new Taq I restriction site.

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6 Nonstandard abbreviations: apo E, apolipoprotein E; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and PCR, polymerase chain reaction.

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Materials and Methods

Subjects. Venous blood samples were obtained from 40 unrelated adult patients participating in an ongoing study of risk factors for coronary heart disease in young or middle-aged persons in the North Carolina area. Six additional patients attending the Lipid Outpatient Clinic of the University Central Hospital of Helsinki were chosen to represent all the known phenotypes of apo E, as determined earlier (17). All subjects were of Finnish origin. We also analyzed blood samples obtained from a Finnish hypertri glyceridemic patient with the apo E phenotype E3/1 (34), his spouse, and three children. For serum lipid measurements, we obtained venous blood samples from this family after 12 h of fasting. Concentrations of cholesterol (35) and triglyceride (36) in serum were determined by enzymatic methods (kits from Boehringer, Mannheim, F.R.G.). The concentration of HDL cholesterol in serum was measured enzymatically after precipitation of LDL and VLDL fractions with dextran sulfate and magnesium chloride (37).

Apo E phenotyping. Apo E phenotyping was carried out by analytical isoelectric focusing on polyacrylamide gel containing ultrapure urea, 8 mol/L, and 2% Ampholine, pH 4–6 (LKB, Bromma, Sweden). For subsequent immunoblotting we used commercially available rabbit anti-human apo E serum (Daichii Pure Chemicals, Tokyo, Japan) (17). We delipidated 4–10 μL of serum with 6 mL of cold ethanol/ethyl ether (3/1 by vol), followed by 3 mL of ethanol/ethyl ether (3/2 by vol); we then dried the sample with ether. The proteins were dissolved in a solution of, per liter, 10 mmol of Tris, 8 mmol of urea, and 200 g of sucrose (pH 8.6). Isoelectric focusing and immunoblotting were then carried out by standard techniques described in detail previously (17).

DNA amplification by the PCR. Leukocytic DNA was extracted from 10 mL of EDTA-anticoagulated whole blood according to Bell et al. (38). We amplified a fragment of the fourth exon of the apo E gene in two steps, using two pairs of oligonucleotide primers (22-mers) designed on the basis of the known nucleotide sequence of the apo E gene (7), as follows:

Primer P1 = 5'-AAGGAGTGGAGGCCTACAAAT-3' (nucleotides 3616–3637)
Primer P2 = 5'-TCGGGCCCCGCGCCGGATCTACA-3' (nucleotides 3914–3893)
Primer P3 = 5'-GAAACACTGAGCCCCGCTGCGG-3' (nucleotides 3643–3670)
Primer P4 = 5'-GGATTGGCGTTAGGCCGGCCTC-3' (nucleotides 3943–3922)

The oligonucleotides were synthesized on a DNA synthesizer (Model 381A; Applied Biosystems, Ramsay, NJ) (39). The template DNA (200 ng per sample) was first amplified by using the primers P1 and P4 (final concentration 1 μmol/L) in a mixture (total volume 100 μL) containing 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP; Tris–HCl (20 mmol/L, pH 8.8 at 22 °C); (NH4)2SO4 (15 mmol/L); MgCl2 (1.5 mmol/L); Tween 20 (1 mL/L); gelatin (0.1 g/L); and 2.5 units of Thermus aquaticus (Tag) DNA-polymerase (United States Biochemicals Corp., Cleveland, OH) in a programmable heat block (PHC-2; Techne Ltd., Cambridge, U.K.) for 25 cycles of 1 min at 96 °C, 1 min at 55 °C, and 1 min at 72 °C. For re-amplification with a nested pair of primers, we transferred to a second PCR an aliquot (3 μL of 100-fold dilution) of the first PCR product amplified with primers P1 and P4. The second PCR was directed by primers P2 and P3 and was carried out at the conditions described above.

Determination of the apo E genotypes by restriction fragment polymorphism analysis. An aliquot (40 μL) of the amplified DNA was digested with the enzyme Hha I (20 U per incubation) for 18 h at 37 °C; no supplementary buffer solution was added to the incubations. The samples were electrophoresed in a 200 g/L polyacrylamide gel for 16 h at 80 V in buffer containing 40 mmol of Tris·HCl (pH 8.05 at 22 °C), 30 mmol of sodium acetate, and 1 mmol of EDTA per liter. After electrophoresis, we submersed the gel for 30 min in the same buffer but containing ethidium bromide, 1 mg/L. The restriction fragments were made visible under ultraviolet light and photographed with a Polaroid camera. To examine the mutation in the patient with the E1 phenotype, we used a similar technique, except that the enzyme Taq I was used instead of Hha I and enzyme digestions were carried out at 65 °C.

Determination of the apo E genotypes by slot–blot hybridization with allele-specific oligonucleotide probes. We diluted 5 μL of amplified DNA with water to 800 μL and added 80 μL of NaOH (3 mol/L). After incubation for 1 h at 60 °C, we added 440 μL of 2 mol/L ammonium acetate reagent. We then blotted the samples in four aliquots (330 μL each) on four different nitrocellulose filters, using Mini- fold II apparatus (Schleicher and Schuell, Dassel, F.R.G.). After baking the samples and filters for 2 h at 80 °C, we prehybridized the samples for 4–6 h at 42 °C in a solution containing, per liter, 0.9 mol of NaCl, 90 mmol of Tris·HCl (pH 7.6 at 22 °C), 1 mmol of EDTA, 5 g of sodium lauryl sarcosine, 0.1 g of transfer RNA, and 1 × Denhardt’s solution (containing, per liter, 0.2 g of Ficoll, 0.2 g of bovine serum albumin, and 0.2 g of polyvinylpyrrolidone). The oligonucleotide probes (19-mers) were designed according to the sequence of the apo E gene (7) as follows (the nucleotide identified in boldface type shows the variable site):

Probe 112-Cys = 5'-GGCGGCGCGACACAGTCTC-3' (nucleotides 3754–3736)
Probe 112-Arg = 5'-GGCGGCGCGACACAGTCTC-3' (nucleotides 3754–3736)
Probe 158-Cys = 5'-CTGCAGAGCSTCTGTGACG-3' (nucleotides 3892–3874)
Probe 158-Arg = 5'-CTGCAGAGCSTCTGTGACG-3' (nucleotides 3892–3874)

We labeled the oligonucleotides with [γ-32P]ATP (Amersham, Bucks, U.K.), using T4 polynucleotide kinase (40). Each of the four filters was hybridized for 20 h at 42 °C in the solution described above, with one of the four labeled oligonucleotides. After hybridization, all the filters were washed twice (5 min each) at room temperature with a solution containing 0.3 mol of NaCl, 30 mmol of trisodium citrate, and 1 g of sodium dodecyl sulfate per liter. Using the same solution, we washed the filters for an additional 2 min at the following temperatures: probe 112-Cys, 67 °C; probe 112-Arg, 73 °C; probe 158-Cys, 61 °C; and probe 158-Arg, 63 °C. For autoradiography, the dried filters were exposed to Kodak-XAR film for 4–24 h at −70 °C.

Results

The strategy of the apo E genotyping based on the Hha I restriction fragment polymorphism of an amplified DNA
fragment is illustrated in Figure 1. During the PCR, the latter pair of amplification primers directs the generation of a DNA fragment 266 nucleotides long. This fragment contains four constant Hha I sites (Figure 1). In addition, Hha I cleaves this fragment whenever a cleavage site (nucleotide sequence GCGC) occurs at codon 112 or 158, e.g., when this codon codes for arginine instead of cysteine.

We analyzed in three different ways six samples known to represent all the known apo E phenotypes (Figure 2). Slot-blot hybridization analysis with allele-specific oligonucleotide probes allowed easy identification of all possible combinations of the three alleles (Figure 2A). In restriction analysis, each of the six genotypes yielded a characteristic restriction fragment pattern in addition to the constant fragments of 63, 18, and 16 bp (Figure 1). The small invariant 18- and 16-bp fragments were too small to be clearly visible in all analyses. The e2/e2 genotype is characterized by the presence of the 91- and 78-bp fragments (Figure 2B). Because in this genotype no Hha I cleavage sites are present at the two polymorphic sites, the 72- and 48-bp fragments are not formed. The genotype e3/e2 shows an additional 48-bp fragment, whereas the genotype e3/e3 lacks the 78-bp fragment (Figure 2B). In the genotype e3/e3, a 72-bp fragment is formed, owing to the presence of the Hha I cleavage site at position 112 (Figure 1 and Figure 2B). In the genotype e4/e3, the 78-bp fragment is absent because both alleles have an arginine residue and thus an Hha I cleavage site at position 158; however, a 91-bp fragment present in the e4/e3 genotype is, in turn, lacking in the genotype e4/e4 (Figure 2B). The 48- and 30-bp fragments are faintly visible in the genotypes that have only one allele containing an arginine residue at position 158 (i.e., genotypes e3/e2 and e4/e2), but are more clearly present in the genotypes with a double dose of arginine at this position (i.e., e3/e3, e4/e3, and e4/e4) (Figure 2B).

Figure 2C illustrates the results from an isoelectric focusing experiment for determining the apo E phenotypes from serum samples from the same individuals.

Aside from the panel of the six samples described above and analyzed by the three different techniques with fully congruent results, we determined the apo E genotypes and phenotypes by the Hha I restriction fragment analysis and isoelectric focusing, respectively, in 40 unrelated individuals. According to the DNA analysis, 22 subjects had the genotype e3/e3, 14 genotype e4/e3, three genotype e3/e2,
and one subject genotype e4/e2. The calculated allele frequencies, based on DNA analysis, were 0.76, 0.19, and 0.05 for e3, e4, and e2, respectively. There was complete agreement between results of the two techniques except that one subject with the genotype e3/e3 (restriction site analysis) was phenotyped as E3/E2 (isoelectric focusing).

Blood samples were also obtained from a patient with a rare apo E phenotype, his spouse, and three children. Earlier studies showed that the patient's apo E isoelectric focusing pattern was characterized by two major bands, one in the E3 position and the other in the E1 position (34). Amino acid sequence analysis of the E1 variant suggested that it differed from E2 at residue 127 by an aspartic acid substituted for glycine, which agrees with the one-charge difference observed in isoelectric focusing (34). The codons for amino acid 126–128 in the normal apo E gene read as CTC GGC CAG (7). Therefore, a Gly→Asp mutation obligatorily implies a GGC→GAC shift in codon 127, which would generate a new Taq I restriction site (recognition sequence TCGA) absent in the normal allele. Phenotyping by isoelectric focusing confirmed the presence of the abnormal apo E3/1 phenotype in the proband (Figure 3A). In this case, the Hha I restriction analysis incorrectly suggested an e3/e2 genotype (Figure 3B). However, as anticipated, the apparent e2 allele of the patient could be distinguished from the normal e2 allele by digestion of the amplified DNA, with the enzyme Taq I cleaving one of the patient's alleles into two pieces that were exactly the predicted size (Figure 3C; cf. Figure 1). This experiment thus confirms the nature of the mutation behind the E1 phenotype. The mendelian inheritance of the mutant apo E allele was demonstrated by analysis of Taq I restriction fragment length polymorphism of the amplified DNA samples from the proband's family members (Figure 4). Analysis of serum lipids confirmed the presence of hypertriglyceridemia in the father; the mother had moderate hypercholesterolemia, the cause of which is unknown (Table 1). One of the children had hypertriglyceridemia, but this did not cosegregate with the abnormal apo E allele present in the family (Figure 4 and Table 1).

Discussion

Genetic polymorphism of apo E has considerable clinical significance because of the association of the e4 allele with increased concentrations of LDL cholesterol in serum (14–17) as well as susceptibility to atherosclerotic vascular disease (20–24). In Finns, the impact of the apo E phenotype is even more pronounced because of the high frequency of e4 allele (23% vs ~15% in most other Western populations) and hypercholesterolemia and coronary artery disease in the population (17, 23). In addition, the e2 allele serves as a genetic marker for type III hyperlipoproteinemia. Although only a small percentage of individuals with the E2/2 phenotype actually express this disease, ~90% of individuals with type III hyperlipoproteinemia have the E2/2 phenotype (3); thus, dietary or hormonal factors may be necessary for the development of the disease.

Apo E phenotypes are routinely determined by use of two-dimensional gel electrophoresis (25) or isoelectric focusing combined with immunoblotting (26). However, these techniques do not permit an exact identification of the molecular alteration of the apo E gene. Methods for DNA analysis initially relied on the use of Southern blot technique, in which the different apo E genotypes were detected after gel electrophoresis with use of allele-specific oligonucleotide probes (41). Development of the PCR technique to amplify the relevant target DNA greatly improved the reliability of the direct apo E genotyping, and size fractionation of the total genomic DNA sample became unnecessary (29–32). The close proximity of the two variable DNA sites of the apo E gene fortunately allows their co-amplification within a single DNA fragment. We have recently introduced two novel detection strategies for apo E genotyping after the initial PCR amplification. In one (42), biotin is introduced into the 5'-ends of the DNA fragments synthesized during the PCR; the fragments are then captured on an avidin-matrix and directly sequenced on this solid support. This technique also permits identification of rare apo E variants caused by DNA mutations of the amino acids 110–160 (3, 4); however, it requires highly skilled personnel and facilities for DNA sequencing. In the second modification, the variable nucleotide in the biotinylated and immobilized DNA is identified by a one-step primer extension reaction directed by a detection step primer that anneals to the DNA immediately upstream of the site of variation (43). In this reaction a single labeled nucleoside triphosphate, complementary to the nucleotide at the variable site, is incorporated. This "mini-sequencing" technique is relatively simple to perform and sensitive enough to allow the use of even tritiated or nonradioactive markers (43).
The amplification of a small defined DNA fragment by PCR has made it possible to use frequently cutting restriction enzymes such as Hha I to identify nucleotide variations. The principle of the present technique, based on the assay of the Hha I restriction fragment length polymorphism of the variable region of the apo E gene, was recently described by Hisson and Vernier (33); however, they provided no comparison with other techniques. The major advantage of this technique is the simplicity of the detection method (polyacrylamide gel electrophoresis), in which no radioactive materials are required. The amplified apo E DNA fragment has a high G + C content, which may decrease the efficiency of the PCR because of the secondary structure of the DNA. We circumvented this problem by carrying out two consecutive PCR amplifications, using a nested set of primers in the second amplification. The nested PCR approach ensures that only the desired specific apo E DNA fragment is amplified and that the amount of DNA generated by PCR is sufficient for detection of the polymorphic fragments by ethidium bromide staining. We also compared the genotyping by restriction enzyme digestion to the slot–blot hybridization technique (29–32), using the prechosen panel of samples with six different genotypes (Figure 2). Although the latter system yielded identical results, it is technically more demanding, and even the slightest deviations of temperature during the final filter–washing step can produce erroneous results.

The disadvantage of the present PCR technique is that rare variants of the apo E gene can be misclassified as one of the normal alleles. Most variants reported are phenotype E2 and involve mutations clustered near residues 140–160 of the apo E molecule; typically, neutral amino acids are substituted for the basic residues arginine or lysine (for a review, see references 3, 4). We found only one discrepant result between data from isoelectric focusing and PCR-assisted genotyping; a sample judged to represent an E3/2 phenotype by the former technique was classified as an E3/e3 genotype by DNA analysis. The fact that the sample was, indeed, typical for apo E3—homozygous for both cysteine at residue 112 and arginine at residue 158—was confirmed by the primer-guided nucleotide incorporation assay (43; data not shown). Whether one of the patient’s alleles has an additional mutation causing an amino acid change and a resulting charge difference of the encoded apo E protein has not been examined. In contrast, we did not observe by isoelectric focusing any deviation of the apo E4 phenotyping from the corresponding restriction site analysis. This strongly suggests that the exceptionally high e4 allele frequency in Finland is due to enrichment of the common e4 genotype in this population.

Our data support Weisgraber et al. (29), who found the apo E phenotypes to match with the cysteine/arginine interchanges at the residues 112 and 158 in all 68 subjects they examined. Funke et al. (41) found a discrepant finding in one of 15 subjects, but their oligonucleotide hybridization technique did not take advantage of the PCR amplification. Emi et al. (31) reported two structural mutants in addition to the common E2 form (Arg1→Cys) among 100 e2 alleles typed by electrophoresis and hybridization with allele-specific oligonucleotide probes. Weisgraber et al. (44) examined 34 apo E2/2 subjects and found all to have the common e2 allele; however, one apo E3/3 control subject proved to have an abnormal e3 allele, functionally equivalent to the e2 allele.

The present study offers one additional example of apo E variants with disagreement between the phenotype and apparent genotype (Figure 3). Our investigation also confirms that this E1 phenotype (34) is due to a single nucleotide change in codon 127 of the apo E gene. The proband is clinically characterized by obesity, eruptive xanthomatosis, mild-to-moderate hypercholesterolemia, and profound hypertriglyceridemia, with no abnormalities of lipoprotein lipase, hepatic lipase, or apolipoprotein CII; the hyperlipidemia has been most responsive to dietary interventions (34). However, it remains unknown whether the hyperlipidemia of the proband is associated with the presence of the E1 phenotype. In fact, this particular family showed no apparent relation between the occurrence of hyperlipidemia and the abnormal apo E allele (Table 1). It is possible, however, that the phenotypic expression of the mutant apo E allele does not become manifest before adult life, which could explain the absence of hyperlipidemia in the son with this gene (Table 1). This study provides a technique whereby the prevalence of this mutant allele in the general population as well as its occurrence among hyperlipidemic subjects can be investigated.

In conclusion, we developed a convenient modification of the PCR-based apo E genotyping techniques. The method is relatively easy to perform and does not require the use of radioisotopes. For routine purposes, it may even replace apo E phenotyping techniques, and for research applications, when combined with apo E phenotyping, it may be useful in the search for genetic heterogeneity among given apo E phenotypes.

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