note on methodology

Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I

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Summary We have used restriction isotyping (restriction enzyme isoform genotyping) for rapid typing of common apolipoprotein E isoforms (E2, E3, E4). ApoE restriction isotyping used oligonucleotides to amplify apolipoprotein E gene sequences containing amino acid positions 112 and 158. The amplification products were digested with *Hha*I and subjected to electrophoresis on polyacrylamide gels. Each of the isoforms was distinguished by a unique combination of *Hha*I fragment sizes that enabled unambiguous typing of all homozygotic and heterozygotic combinations. *Hha*I cleaves at GCGC encoding 112arg (E4) and 158arg (E3, E4), but does not cut at GTGC encoding 112cys (E2, E3) and 158cys (E2). —**Hixson, J. E., and D. T. Vernier.** Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. *J. Lipid Res.* 1990. **31:** 545–548.

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Apolipoprotein E (apoE) plays a central role in cholesterol transport as reflected by the association of apoE with a variety of lipoprotein size classes, its synthesis in a variety of different tissues, and the ability of apoE to interact with two distinct hepatic receptors (LDL receptor and apoE receptor) (1). Transport of cholesterol from peripheral tissues to the liver (reverse transport) may be accomplished via binding of apoE containing HDL particles to hepatic apoE receptors for catabolism (1). The human apoE gene spans 3.7 kb including four exons (2, 3) and is located on chromosome 19 (3) in a gene family that also contains the genes for apoC-I, C-I' (a pseudogene), and C-II (4).

Genetic variation at the apoE locus in human populations is an important determinant of plasma lipid levels and relative risk of atherosclerosis (5). Three common alleles of apoE encoding isoforms E2, E3, and E4 (6, 7) and several rare variants have been identified (1). E3 is the most common of these isoforms, and is distinguished by cysteine at position 112 (112cys) and arginine at position 158 (158arg) in the receptor-binding region of apoE (8). The E4 isoform (112arg and 158arg) is associated with increased levels of total cholesterol and betalipoprotein (9), and increased susceptibility to heart disease (5, 10). Most patients with type III hyperlipidemia are homozygous for the E2 isoform (112cys and 158cys) that binds with reduced affinity to cellular receptors (11, 12). In population studies, the E2 isoform is associated with decreased levels of cholesterol and betalipoprotein (9).

These reports of significant effects of apoE genotype on heart disease and known risk factors of heart disease have led to increased interest in rapid typing of apoE isoforms for population studies. Recently, methods were developed for apoE typing at the DNA level using genomic DNA for Southern blots with allele-specific oligonucleotide (ASO) probes that span positions 112 and 158 (13, 14). The sensitivity of this method was increased by using the polymerase chain reaction (PCR, 15) to amplify apoE sequences for dot-blots with ASO probes (16). In addition, automated sequence analysis of PCR products has been used for typing common and rare apoE isoforms (16). In this report, we describe restriction isotyping (restriction enzyme isoform genotyping) as a simpler and faster method for typing the common apoE isoforms. Restriction isotyping uses PCR amplification, but avoids the use of costly and time-consuming hybridization and sequencing techniques. After amplification of apoE sequences that encompass amino acid positions 112 and 158, we simply digest the PCR products with *Hha*I and separate the resulting digestion fragments by electrophoresis on polyacrylamide gels. Because the nucleotide substitutions that result in arg-cys interchanges at positions 112 and 158 also alter *Hha*I cleavage sites (17), each genotype can be distinguished by unique combinations of *Hha*I fragment sizes in all homozygotic and heterozygotic combinations.

Restriction isotyping relies on amplification of discrete genic regions enabling use of restriction enzymes that cut frequently (recognition sequences of 4 bp). These enzymes survey a larger proportion of a given sequence for cleavage sites, but produce a large number of small DNA fragments that have not been suitable for Southern blot analyses of complex genomes. In addition, in vitro amplified DNA is not methylated, allowing the use of a wide variety of restriction enzymes. For example, methylation may have prevented *Hha*I cleavage of genomic DNA for Southern blots in a previous study of apoE (17). For these reasons, restriction isotyping may be useful as a general approach where substitutions alter cleavage sites that distinguish particular isoform genotypes, or for detection of unknown variants in surveys with many different enzymes.

Abbreviations: apoE, apolipoprotein E; ASO, allele-specific oligonucleotide; bp, base pairs; LDL, low density lipoprotein; PCR, polymerase chain reaction.

MATERIALS AND METHODS

Amplification of apoE sequences from genomic DNA for restriction isotyping

Leukocyte DNA was extracted as previously described (18) from subjects who were previously typed with respect to apoE isoforms by isoelectric focusing of serum proteins (leukocytes provided by Dr. Craig Hanis). Leukocyte DNA was amplified by PCR in a DNA Thermal Cycler (Perkin Elmer Cetus) using oligonucleotide primers F4 (5'-ACAGAATTCGCCCCGGCCTGGTACAC-3') and F6 (5'-TAAGCTTGGCACGGCTGTCCAAGGA-3') described by Emi et al. (16). In addition to the buffer and nucleotide components described by the supplier of *Taq* polymerase (Perkin Elmer Cetus, 15), each amplification reaction contained 1 μg of leukocyte DNA, 1 pmol/μl of each primer, 10% dimethyl sulfoxide, and 0.025 units/μl of *Taq* polymerase in a final volume of 30 μl. Each reaction mixture was heated at 95°C for 5 min for denaturation, and subjected to 30 cycles of amplification by primer annealing (60°C for 1 min), extension (70°C for 2 min), and denaturation (95°C for 1 min). We obtained approximately 300 ng of amplified apoE sequences from each amplification reaction. Amplification of apoE products that were suitable for *Hha*I digestion proved successful for most DNA samples, with the exception of samples that were extensively degraded prior to amplification.

Restriction isotyping of amplified apoE sequences with *Hha*I and gel analysis

After PCR amplification, 5 units of *Hha*I (New England Biolabs) were added directly to each reaction mixture for digestion of apoE sequences (>3 h at 37°C). This process did not require purification of PCR products or addition of specific buffer components for *Hha*I digestion. Incomplete digestion was not a significant problem, most likely due to the large amounts of *Hha*I enzyme relative to the small amount of amplified sequences in each reaction (5 units *Hha*I/300 ng DNA). Each reaction mixture was loaded onto an 8% polyacrylamide nondenaturing gel (1.5 mm thick × 25 cm long) and electrophoresed for 3 h under constant current (45 mA). After electrophoresis, the gel was treated with ethidium bromide (0.2 mg/l) for 10 min and DNA fragments were visualized by UV illumination. The sizes of *Hha*I fragments were estimated by comparison with known size markers (*Msp*I-digested pUC18 DNA).

RESULTS AND DISCUSSION

ApoE restriction isotyping by PCR amplification and cleavage with *Hha*I

ApoE restriction isotyping relies on cleavage at polymorphic *Hha*I sites to distinguish E2, E3, and E4 sequences, rather than on hybridization of Southern blots

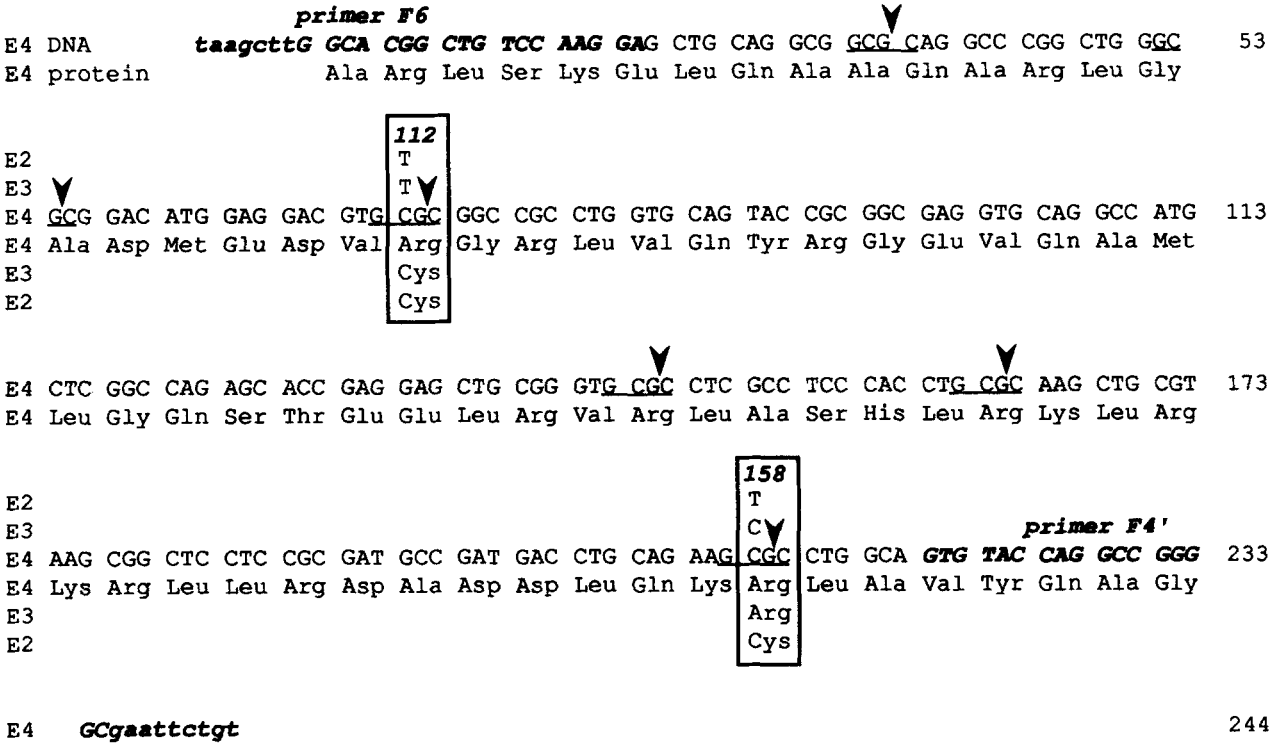

Fig. 1. DNA and protein sequences of amplified regions encoding common apoE isoforms and locations of *Hha*I cleavage sites. The amplified E4 nucleotide sequence (244 bp, numbered to the right) is shown above the E4 amino acid sequence. The sequences of amplification primers (F6 and F4', the reverse complement of F4) are also shown (upper case italics are apoE sequences, lower case italics are synthetic cleavage sites). Nucleotide substitutions that distinguish E2 and E3 isoforms are shown above the E4 nucleotide sequence, and amino acid substitutions are shown below the E4 amino acid sequence (substitution sites at codons 112 and 158 are boxed). The sites for *Hha*I cleavage in the E4 nucleotide sequence are underlined and marked by arrows.

or dot-blots with ASO probes. The first step is amplification of apoE sequences containing amino acid positions 112 and 158 from genomic DNA. **Fig. 1** shows the sequence (244 bp) encoding the E4 isoform after amplification by PCR with F4 and F6 primers (16). The second step is cleavage of amplified apoE sequences with *Hha*I for polyacrylamide gel electrophoresis. Fig. 1 shows the six *Hha*I cleavage sites (GCGC) in the amplified E4 sequence (2), including *Hha*I sites at codons for arginine residues (GCGC) at positions 112 and 158. The nucleotide and amino acid substitutions that distinguish and E2 and E3 isoforms are aligned with the E4 sequence in Fig. 1. The E3 sequence encodes a cysteine residue at position 112 (GTGC) which abolishes the *Hha*I cleavage site in the E4 sequence, resulting in a total of five *Hha*I cleavage sites. The E2 sequence encodes cysteines at positions 112 (GTGC) and 158 (GTGC) that abolish two cleavage sites relative to the E4 sequence, resulting in a total of four *Hha*I cleavage sites.

Fig. 2 shows gel-separated products of apoE amplification and *Hha*I digestion using leukocyte DNA from human subjects representing each homozygotic and heterozygotic combination of common apoE alleles. Fig. 2 also shows *Hha*I cleavage maps for each isoform, and the fragment sizes from polymorphic *Hha*I sites. With the exception of a shared 38 bp fragment (common *Hha*I site at position 38 in Fig. 1, the other shared fragments were not detected due to their smaller sizes), each genotype possessed unique combinations of *Hha*I fragment sizes. The E2/E2 sample contained 91 and 83 bp *Hha*I fragments reflecting the absence of sites at 112cys and 158cys. The E3/E3 sample also contained the 91 bp fragment (112cys), as well as 48 and 35 bp fragments from cleavage at the *Hha*I site at 158arg. The E4/E4 sample also contained these 48 and 35 bp fragments (158arg), as well as a unique 72 bp fragment from cleavage at 112arg (the 19 bp fragment was too small for detection). Each of the samples from heterozygotic combinations contained both sets of fragments from each apoE allele.

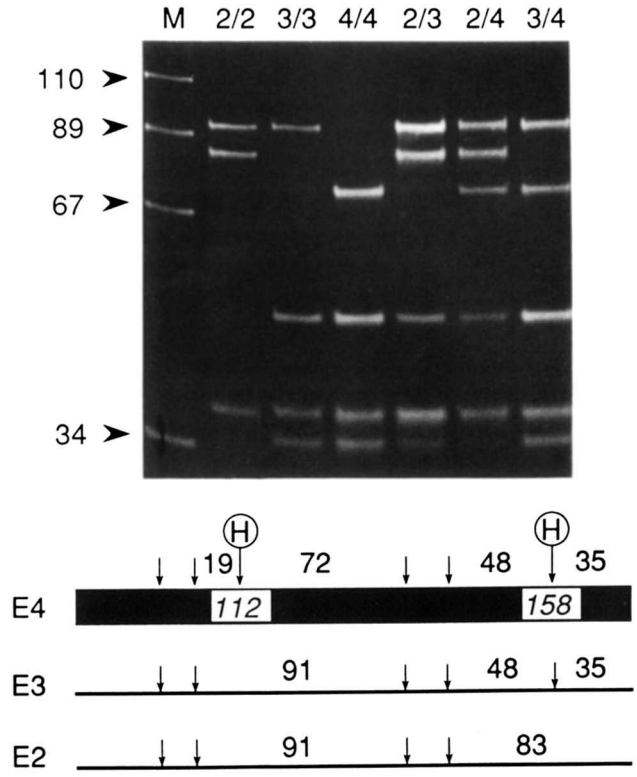

Fig. 2. *Hha*I cleavage maps and electrophoretic separation of *Hha*I fragments after gene amplification of DNA from subjects with known apoE isoforms. At the top, a polyacrylamide gel is shown after electrophoresis of *Hha*I fragments from an E2E2 homozygote (lane marked 2/2), E3E3 homozygote (3/3), E4E4 homozygote (4/4), E2E3 heterozygote (2/3), E2E4 heterozygote (2/4), and E3E4 heterozygote (3/4). The fragment sizes (in bp) of a DNA standard (*Msp*I-digested pUC18, lane marked M) are shown to the left of the gel. Below the gel, *Hha*I cleavage maps (downward arrows show sites) are given for amplified sequences (E4 is shown as a filled box containing codons 112 and 158, E3 and E2 maps are shown below E4). The distances (in bp) between polymorphic *Hha*I sites (circled H) that distinguish isoforms are shown for each cleavage map.

Advantages of apoE restriction isotyping

Recent advances in molecular technologies have allowed genotyping of apoE isoforms by direct detection of nucleotide substitutions in genomic DNA rather than isoelectic focusing of serum isoforms (13, 14, 16). The use of ASO probes for apoE genotyping relies on the increased stability of homoduplexes formed by allele-specific probes with corresponding allelic sequences, relative to heteroduplexes with alternative ASO probes that differ by a single substitution (13). In that method, genomic (13, 14) or PCR-amplified (16) DNA is subjected to Southern blots (13, 14) or dot-blots (16) followed by separate hybridizations with each of four radiolabeled oligonucleotides to distinguish 112cys, 112arg, 158cys, and 158arg. After hybridization, high stringency washes at critical temperatures and NaCl concentrations are used to remove probes with single mismatches.

While ASO probes do distinguish apoE genotypes, obtaining results for large population studies by hybridization of each sample with four different oligonucleotides is extremely time-consuming and technically demanding relative to restriction isotyping. The use of ASO probes for dot-blots is particularly sensitive to nonspecific binding or background radioactivity, so that a particular sample often requires multiple testing to obtain unambiguous hybridization patterns. In contrast, each genotype is readily determined by the presence of specific *Hha*I fragment sizes after electrophoresis (Fig. 2). In addition, *Hha*I apoE genotypes are visualized directly by gel staining that does not require radionuclides that are typically used to label ASO probes.

Another technique that has been described to detect apoE genotypes uses automated DNA sequencing of amplified apoE genes (16). The advantage of that approach is that rare variants can be identified that are not

detected by isoelectric focusing or ASO probes in hybridization assays. In contrast to sequencing, our method cannot detect rare variants, unless substitutions alter *Hha*I sites within the amplified region of apoE. However, sequencing apoE genes to detect rare variants may not be a practical alternative for many laboratories conducting large population studies to identify apoE gene effects.

Restriction isotyping as a general method for detecting polymorphisms

Most methods for detection of allelic variation have relied on protein electrophoresis or nucleic acid hybridization with particular oligonucleotide or gene probes (19). Restriction isotyping may provide a simpler alternative to these methods by enabling detection of substitutions using restriction enzymes that cut more frequently (4 bp recognition sites) and survey a large proportion of a given sequence. Restriction isotyping uses in vitro amplified DNA, allowing analyses of small fragments produced by these enzymes, and cleavage of sequences that are methylated in genomic DNA.

Restriction isotyping may be applied for variants with known amino acid or nucleotide substitutions that predict the use of a specific restriction enzyme (*Hha*I for apoE isoforms), or for detection of unknown polymorphisms using surveys of many different restriction enzymes. In addition, restriction isotyping used in conjunction with protein phenotyping may identify rare alleles that mimic particular protein variants. For example, rare E2 mutations may be identified by discrepancies between isoelectric focusing that detects E2-migrating isoforms in a particular individual, and restriction isotyping that does not detect the prevalent E2 allele (112cys, 158cys) in the same individual.

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