Analysis of Apolipoprotein E Genotypes by the Amplification Refractory Mutation System

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The Amplification Refractory Mutation System (ARMS) has been successfully applied to the detection of apolipoprotein (apo) E genotypes in human DNA extracted from peripheral blood. By using four allele-specific oligonucleotide primers and one common primer, one can identify the three common alleles of the apo E genetic polymorphism, ε2, ε3, and ε4. The system amplifies two sequences of the apo E gene, one of 181 bp and the other 319 bp. These sequences are amplified when DNA containing a particular allele is incubated with its allele-specific oligonucleotide primer and a common primer. The method is simple, reliable, and nonisotopic and obviates the need for digestion with restriction endonucleases or for hybridization with allele-specific oligonucleotide probes. Genotyping DNA by this method overcomes the problem of post-translational modification of the apo E phenotype encountered with isoelectric focusing of the mature plasma apo E protein.

Additional Keyphrases: polymerase chain reaction • DNA probes • genetic variants

Apolipoprotein (apo) E, a protein constituent of the triglyceride-rich lipoproteins, very-low-density lipoprotein (VLDL), and chylomicrons, is a single-chain polypeptide of 299 amino acids. The apo E gene has been mapped to chromosome 19 (1, 2), and its nucleotide sequence and structure have been determined (3). Apo E is thought to play a central role in lipoprotein metabolism. Mahley (4) proposes that chylomicron and VLDL remnant particles are both cleared from the circulation by hepatic apo E receptors.

The apo E gene is polymorphic. The three common alleles—ε2, ε3, and ε4—are inherited co-dominantly and code for three apo E proteins (isofoms): E2, E3, and E4. The isofoms differ at amino acid residues 112 and 158. Isoform E3 has cysteine residues at both sites, E4 has arginine residues at both sites, and E2 has a cysteine at position 112 and an arginine at position 158 (5). The substitutions produce charge differences, allowing for isoform separation by isoelectric focusing.

In epidemiological studies, apo E4 has been associated with concentrations of apo B and low-density lipoprotein cholesterol that are higher than those associated with apo E2, which in turn are higher than those associated with apo E3. It has therefore been proposed that the apo E genotype may confer a susceptibility to atherosclerosis and to coronary heart disease (6).

Apo E2 has a lower affinity for cellular receptors than does either apo E3 or apo E4 (7). Consequently, individuals homozygous for the ε2 allele have a predisposition to accumulate lipoprotein remnants in the circulation; however, a clinically significant accumulation, known as Type III hyperlipidemia (6), occurs in only about 5% of such homozygotes. These patients generally have other conditions such as diabetes, hypothyroidism, or obesity, which predispose to hyperlipidemia. They have a combined hyperlipidemia with increased blood concentrations of cholesterol and triglyceride. The presence of the ε2ε2 genotype establishes the diagnosis of Type III remnant hyperlipidemia, a disorder associated with accelerated atherogenesis and most appropriately treated with diet and the fibrate-class of drugs (8). Recently, Nestruck et al. (9) suggested that apo E may modulate the response of lipoproteins to drug therapy.

Early methods for detecting the apo E genotype were based on phenotype assignment after isoelectric focusing of VLDL or serum (10, 11). More recently, to increase specificity, isoelectric focusing has been combined with immunoblotting with specific anti-apo E antisera (12, 13). These methods still require pretreatment of samples with neuraminidase to remove sialic acid residues from the apo E proteins. Even then, correct phenotype assignment may be rendered difficult by other post-translational modifications, e.g., nonenzymatic glycation of apo E, as occurs in diabetes mellitus (14–16), or artefacts caused by the prolonged storage of sera (13).

After the sequencing of the apo E gene (3) and the recognition of the base substitutions responsible for the polymorphisms, these problems have been eliminated by methods that determine genotype directly by using the polymerase chain reaction (PCR) and hybridization with radiolabeled oligonucleotide probes (17–19). However, these techniques involve the use of radiation and are not well-suited for the rapid processing of large numbers of samples.

The Amplification Refractory Mutation System (ARMS) extends the PCR to allow the rapid analysis of known mutations in genomic DNA. The genotype is determined by agarose gel electrophoresis of the reaction mixtures (20). In this system we use an oligonucleotide specific for the mutation site as one of the PCR primers. The technique is based on the observation that if a primer is mismatched at the 3'-nucleotide, amplification will not occur. The allele-specific primer is therefore synthesized in two forms, one with the mutant and
the other with the nonmutant 3'-nucleotide. Amplification will occur with the mutant primer if the mutation is present in the template DNA, or with the nonmutant primer if the mutation is absent, so that hybridization with labeled allele-specific oligonucleotides is unnecessary. To ensure the specificity of the primers, we introduce a deliberate base mismatch adjacent to the 3' nucleotide (see below).

In the present investigation we have applied the ARMS concept to the determination of apo E genotypes.

Materials and Methods

DNA preparation. DNA samples were obtained by the method of Kunkel et al. (21) from peripheral blood from 51 subjects of known apo E genotype with the following frequency distribution: $e_2e_2$ (n = 1), $e_2e_3$ (23), $e_3e_3$ (1), $e_4e_4$ (10), $e_4e_3$ (1), $e_4e_2$ (4). The genotype had been determined previously by PCR and hybridization with allele-specific oligonucleotide probes by the method of Houston et al. (17).

Oligonucleotide primers. The oligonucleotide primers were prepared with a Model 380A DNA synthesizer (Applied Biosystems, Ramsey, NJ) and used without further purification. The orientation of the allele-specific and common primers within the apo E gene is illustrated in Figure 1. The base sequence of the allelespecific ARMS $e_2$ primer used to detect the variation at nucleotide 3884 (amino acid 158) was 5’TCCGCCG-GATGCCGATGACCTGCAGAATF3’, and that of the $e_3$ primer was 5’TCCGCCGATGCGATAGCTGCAGAATTT3’. The base sequence of the allele-specific ARMS $e_4$ primer used to detect the variation at nucleotide 3746 (amino acid 112) was 5’CCCGGC-TGGGCGCGCACTGGAGGCACGTC3’; that of the $e_3$ primer was 5’CCGCGCTGGGCGCGCAGATGGACCGAGACAC3’. The underlined base, next to the allele-specific 3'-base, is the base that has been deliberately destabilized by substituting T for G to ensure absolute allele-specificity. The common primer was 5’TGGGC-CCGCTCCTGTAGCGGCTGCGCCGCGCC3’.

After the conditions necessary to achieve successful allele-specific amplification of the ARMS primers alone were established, we tested several possible internal control primers for amplification under the same conditions. These spanned (a) a 360-bp fragment of exon 3 of the $\alpha_1$-antitrypsin gene, (b) a 220-bp region of exon 5 of the $\alpha_1$-antitrypsin gene, and (c) a 510-bp region of exon 26 of the apo B gene. Only those primers spanning the 360-bp fragment of the $\alpha_1$-antitrypsin gene co-amplified with the ARMS primers (Figure 2); these were therefore selected as the internal control primers (22). These primers produce an internal control amplification product whether or not there is amplification with the ARMS primers.

ARMS analysis of genomic DNA. Four reactions were set up for each sample. Each of the four ARMS primers was used with the common primer and the internal control primers. All reactions were carried out with 1 $\mu$g of genomic DNA and 1 unit of Taq DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Beaconsfield, Bucks., U.K.) in a volume of 50 $\mu$L. The final concentrations of other constituents were, per liter, 10 mmol of Tris (pH 8.3), 1.5 mmol of MgCl2, 50 mmol of KCl, 0.1 g of gelatin, 200 $\mu$mol of all four deoxynucleoside triphosphates, 1 $\mu$mol of each of the ARMS and internal control primers, and 100 mL of dimethyl sulfoxide. The reaction mix was overlaid with 50 mL of paraffin oil. The first step in DNA synthesis consisted of denaturation at 94°C for 5 min and primer annealing at 60°C for 1 min, followed by 40 cycles of primer extension at 64°C for 5 min, denaturation at 94°C for 0.5 min, and primer annealing at 60°C for 1 min. Finally, there was one cycle of primer extension for 10 min at 64°C. Reactions were carried out with an HB TR1 Thermal Reactor (Hybaid Ltd., Teddington, Middlesex, U.K.).

Detection of amplification products. We added 2 $\mu$L of Orange G solution, 0.1 g/L in 300 g/L Ficoll solution (molecular mass 400 kDa), to 20 $\mu$L of reaction mixture and electrophoresed for 6 h at 80 V on a 3% agarose gel containing, per liter, 15 g of agarose and 15 g of NuSieve GTG (ICN Biomedicals, High Wycombe, U.K.). The amplified DNA fragments were made visible by staining with

![Fig. 1. Relative positions of the ARMS and common primers within the apo E gene](image)

The bases are numbered after Paik et al. (3)

![Fig. 2. Agarose gel electrophoresis of PCR products after amplification of DNA from a subject with genotype $e_2e_2$ in the absence and presence of the internal control primers spanning a 360-bp fragment of exon 3 of the $\alpha_1$-antitrypsin gene](image)

Tracks are as follows: 1 and 11, 123-bp ladder; 2 and 7, Arg 158 ARMS primer; 3 and 8, Cys 158 ARMS primer; 4 and 9, Cys 112 ARMS primer; 5 and 10, Arg 112 ARMS primer; 6, internal control primers alone. Tracks 2–5, without internal control primers; tracks 7–10, with internal control primers.
ing the gel after electrophoresis with ethidium bromide for viewing through an ultraviolet transilluminator (U.V. Products Inc., Cambridge, U.K.). A molecular mass marker was included in each gel (123-bp ladder; Gibco BRL, Paisley, U.K.) to confirm the size of amplified fragments.

Results

Amplification of samples of known genotype. The observed and expected ARMS results obtained from 50 samples previously genotyped by the method of Houlston et al. (17) are summarized in Table 1. In one sample, no amplification occurred with either the ARMS or the internal control primers. All the other samples gave the correct amplification patterns expected of a particular genotype. Each of the three homozygotes has two different amplification products. In $\epsilon_2\epsilon_2$ samples, these were obtained with primers Arg 158 and Cys 112 (Figure 2, tracks 7–10). In $\epsilon_2\epsilon_3$ samples they were obtained with primers Cys 158 and Cys 112 (Figure 3, tracks 2–5), and in $\epsilon_4\epsilon_4$ samples they were obtained with primers Arg 158 and Arg 112 (Figure 3, tracks 6–9). One of the heterozygotes, $\epsilon_4\epsilon_2$, has an amplification product with each of the four primers (Figure 4, tracks 2–5). The other two have three products, $\epsilon_2\epsilon_3$ with all but the Arg 112 primer (Figure 4, tracks 6–9), and $\epsilon_4\epsilon_3$ with all but the Cys 158 primer (Figure 4, tracks 10–13). ARMS therefore successfully distinguishes between the six genotypes and the corresponding protein phenotype.

Discussion

ARMS was first described when it was used to detect mutations in the $\alpha_1$-antitrypsin gene (20). Subsequently the technique has been used in the prenatal diagnosis of cystic fibrosis (23, 24) and phenylketonuria (25), and is extended here to the direct analysis of apo E genotypes. ARMS has several advantages over the indirect method of analysis in which genotype is inferred from protein isoforms and where artefacts may be produced by post-translational modification of apo E; e.g., apo E may be glycosylated as much as 30-fold in patients with diabetes (14), which increases the anodal mobility of the protein and may lead to a misclassification of genotype with an apparent increase in $E_2$ and decrease in $E_4$ frequency. This might explain the increased incidence of apo $E_2$ in patients with insulin-dependent diabetes compared with a healthy population (26). Another advantage of the ARMS system is that DNA, unlike protein, is stable indefinitely when stored at $-20^\circ$C, so this technique can be applied retrospectively to the analysis of DNA samples that have been stored for a considerable time.

The other DNA methods for the detection of apo E genotypes by DNA analysis make use of hybridization with ASO probes, with or without PCR (17–19, 27), but these involve several stages, including isotopic labeling of an oligonucleotide probe. In contrast, ARMS analysis of DNA involves only two stages: PCR and agarose gel electrophoresis. For batch analysis, the procedure may be simplified further by analyzing the samples one

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of subjects</th>
<th>$\text{Arg 158}$</th>
<th>$\text{Cys 158}$</th>
<th>$\text{Cys 112}$</th>
<th>$\text{Arg 112}$</th>
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<td>$+/+$</td>
<td>$+/+$</td>
<td>$-/-$</td>
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<td>$-/-$</td>
<td>$+/+$</td>
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<tr>
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<td>$+/+$</td>
<td>$-/-$</td>
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<td>$+/+$</td>
</tr>
<tr>
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<tr>
<td>$\epsilon_4\epsilon_2$</td>
<td>4</td>
<td>$+/+$</td>
<td>$+/+$</td>
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<td>$+/+$</td>
</tr>
</tbody>
</table>

Fig. 3. ARMS analysis of subjects of genotype $\epsilon_2\epsilon_2$ (tracks 2–5) and $\epsilon_4\epsilon_4$ (tracks 6–9) ARMS primers are as follows: 2 and 6, Arg 158; 3 and 7, Cys 158; 4 and 8, Cys 112; 5 and 9, Arg 112. Tracks 1 and 10, 123-bp ladder

Fig. 4. ARMS analysis of subjects of genotype $\epsilon_4\epsilon_2$ (tracks 2–5), $\epsilon_2\epsilon_2$ (tracks 6–9); and $\epsilon_4\epsilon_3$ (tracks 10–13) ARMS primers are as follows: 2, 6, and 10, Arg 158; 3, 7, and 11, Cys 158; 4, 8, and 12, Cys 112; 5, 9, and 13, Arg 112. Tracks 1 and 14, 123-bp ladder
allele at a time, by pipetting a master mix of reagents, water, buffer, deoxynucleoside triphosphates, primers, and enzyme into a series of tubes; aliquots of the target DNA samples are then added to the tubes. This approach saves both time and pipette tips.

The ARMS concept depends on the absence of a 3′-exonucleolytic proofreading activity associated with the Taq DNA polymerase (20, 28, 29). This prerequisite, which has been discussed elsewhere (20), has been confirmed here by another successful application of ARMS. ARMS also requires that the 3′-OH-terminal mismatched primers are refractory to extension by the DNA polymerase. The degree of refractoriness depends on the type of the mismatch (20). The purine/purine or pyrimidine/pyrimidine mismatches A/A, C/T, T/T, and C/C are more refractory to amplification than are the purine/pyrimidine mismatches G/T and A/C. A 3′-nucleotide purine/pyrimidine mismatch therefore requires additional destabilization to ensure primer specificity. We found that the ARMS primers generated a G/T or A/C primer/template mismatch. Therefore, we introduced additional destabilization by substituting the T for the G adjacent to the 3′-nucleotide in each of the four primers, to generate C/T mismatches. Specificity was further enhanced by using large (30-mer) primers with a GC content of >50%. A relatively high annealing temperature of 60 °C also reduced nonspecific binding of primers.

The analysis of apo E genotypes by ARMS is both reliable and robust. Only one DNA sample failed to amplify at all, perhaps because of contamination of the DNA with EDTA, as has been reported by others (25). However, we were unable to pursue this hypothesis because of a shortage of sample. In conclusion, we find that, with good-quality DNA samples, ARMS is a reliable method of phenotyping apo E and probably preferable to phenotyping by isoselective focusing of proteins for studying patients with diabetes or patients in whom phenotypic assignment by traditional procedures is equivocal.

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References