Time-resolved fluorometry in the genetic diagnosis of familial defective apolipoprotein B-100

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Summary A novel technique for screening point mutations has been developed for diagnosis of familial defective apolipoprotein (apo) B-100 (FDB). In FDB, an amino acid exchange occurs at position 3500 in apoB-100 due to a point mutation. Polymerase chain reaction (PCR) was performed on the appropriate region of the apoB gene, and the PCR products were hybridized in solution with europium-labeled oligonucleotides, complementary to either the wildtype or the mutant genome. The presence or absence of the apoB-3500 mutation was monitored by time-resolved fluorescence of the europium chelate. The method allows a larger number of samples to be processed simultaneously, and the detection system displays a high level of sensitivity without the hazards connected to the use of radioactivity. When 127 Swedish patients, clinically diagnosed as suffering from heterozygous familial hypercholesterolemia, were screened for the presence of the apoB-3500 mutation, two patients, unrelated to each other, were found to be heterozygotes. These patients are the first reported cases of FDB from Sweden, and the frequency rate observed among hypercholesterolemic patients, 1.6%, is in accordance with the figures reported for several other patient populations in Europe and the United States. **—Eggertsen, G., M. Eriksson, O. Wiklund, A. Iitiä, S-O. Olofsson, B. Angelin, and L. Berglund.** Time-resolved fluorometry in the genetic diagnosis of familial defective apolipoprotein B-100. *J. Lipid Res.* 1994. **35:** 1505–1508.

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Two genetic disorders of lipoprotein metabolism, familial hypercholesterolemia (FH) and familial defective apolipoprotein (apo) B-100 (FDB), affect the interaction between the low density lipoprotein (LDL)-receptor on tissue cells and its proper ligands on plasma lipoproteins, apoB-100 and apoE (for a review see references 1–3). In FH, a large number of various mutations occur in the LDL-receptor, while in FDB only a single point mutation has been found in apoB-100 corresponding to amino acid position 3500 (apoB-3500) (4, 5). Both FH and FDB occur as autosomal dominant diseases. Presently it is difficult to differentiate between the two diseases in patients with hypercholesterolemia without using techniques based on molecular biology as the two diseases may produce identical symptoms.

There is an increasing demand for accessible techniques useful for diagnostics of genetic disorders. Available techniques now allow direct assaying of structural defects in the genome, but, in addition, appropriate methods should permit larger numbers of specimens to be processed in a convenient way. Here we describe a solution hybridization technique that utilizes a non-radioactive detection system, based on time-resolved fluorescence with the lanthanide (Eu^{3+}) chelates (6). This technique was used to identify the first two patients with the apoB-3500 mutation in Sweden.

MATERIAL AND METHODS

Patients

The study comprised a total of 127 patients, 63 males and 64 females, with a clinical diagnosis of heterozygous familial hypercholesterolemia. They were recruited from the lipid clinics of Huddinge University Hospital in Stockholm (52 patients) and Sahlgren's Hospital in Göteborg (75 patients). A detailed presentation of the criteria for diagnosis and the clinical data of the participants have been reported previously (7). For comparison, a number of samples were obtained from healthy volunteers of both sexes. Genomic DNA from a 43-year-old woman with established FDB, due to heterozygosity for the apoB-3500 mutation (8), was kindly supplied by Professor Steve Humphries (Center for Genetics of Cardiovascular Disorders, University College of London School of Medicine, London, UK) and analyzed in parallel with our own samples in the assay.

Materials

The following items were purchased: Taq-polymerase (Perkin-Elmer Cetus Instruments, Emeryville, CA); phenol (IBI, New Haven, CT); proteinase K (E. Merck, Darmstadt, Germany); Delfia Assay Buffer, Delfia Enhancement Solution, streptavidin-coated microtiter plates (Wallac Oy, Turku, Finland); Dynabeads M-280 (Dynal A. S., Olso, Norway).

Abbreviations: FDB, familial defective apolipoprotein B-100; PCR, polymerase chain reaction; apoB, apolipoprotein B; FH, familial hypercholesterolemia; LDL, low density lipoprotein; cps, counts per second; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

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Europium-labeled (Eu^{3+}) oligonucleotides (WO 68, 5' $(NH_2\text{-}C)_{15}$GC ACA CGG TCT TC 3'), and WO 69, 5' $(NH_2\text{-}C)_{15}$AGC ACA CAG TCT TC 3'), and a biotinylated oligonucleotide (WO 66, 5'CTG CAG GGC ACT TCC AAA ATT GAT $(NH_2\text{-}C)_1$A) were synthesized at Wallac Oy, Turku, Finland, according to previously published methods (9). Briefly, the oligonucleotides were prepared on a Gene Assembler oligonucleotide synthesizer (Pharmacia Biotechnology, Piscataway, NJ), deprotected and purified according to standard procedures. A diaminohexane-modified deoxycytidine phosphoramidite was used during the oligonucleotide synthesis to introduce reactive sites for the labeling of the probes with either europium (10) or biotin. After insertion of diaminohexane-modified deoxycytidines in the 5'-end of WO 68 and WO 69, the oligonucleotides were labeled with the Eu^{3+}-chelate W2014, which uses isothiocyanate as its reactive group. The labeled products were purified by gel filtration. The content of (Eu^{3+}) was determined by time-resolved fluorometry, and each oligonucleotide was found to contain 15 (Eu^{3+}) chelates in the 5'-end. The probe WO 66 was biotinylated using biotin-aminocaproate N-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO).

Analytical procedure

The procedure is outlined in **Fig. 1.** Genomic DNA was prepared from peripheral white blood cells by phenol–chloroform extraction of proteinase K-treated nuclei in microscale as described (11). Amplification of a 345 basepair fragment including the putative mutation spot (nucleotide no. 10699 of the cDNA) in exon 26 was performed as outlined by Tybjaerg-Hansen et al. (8). Amplification was carried out using 92°C/30 sec for denaturation, 59°C/30 sec for annealing, and 72°C/160 sec for extension during 25 cycles. For the hybridization reaction, two europium-labeled oligonucleotides were used (see Fig 1.), one complementary with the wildtype (WO 68) and one with the mutant gene (WO 69), corresponding to nucleotides no 10693 to 10705 in the cDNA (12). In addition, a biotinylated "universal" oligonucleotide (WO 66) was used, which was complementary to a region in the 3'-part of the PCR fragment outside the binding region of the other two oligonucleotides (corresponding to nucleotides 10713–10736 in the cDNA (12)). The amplified material was denaturated by incubation at 100°C for 10 min and then put on ice. To 10 μl of the denaturated PCR reaction, approximately 1 ng of either of the two europium-labeled oligonucleotides was added together with 1 ng of the universal (biotinylated) oligonucleotide (WO 66) and 100 μl of hybridization buffer (Delfia Assay Buffer with 1 M NaCl). After vortexing, the tubes were incubated for 35°C for 1 h. The specimens were then transferred to streptavidin-coated microtiter plates, and 100 μl of Delfia Assay Buffer was added per well, whereafter the plates were shaken gently on the Delfia Plateshake (Wallac Oy) for 1 h at room temperature. The plates were washed 6 times with wash solution at 35°C, 200 μl of Delfia Enhancement Solution was added, and the plates were put in the Delfia Plateshake for another 25 min at room temperature. Finally, the fluorescence was monitored in a 1232 Delfia TR-Fluorometer (Wallac Oy) at a wavelength of 613 nm.

Direct sequencing of the PCR products was carried out using the Sequenase kit (USB, Cleveland, OH) on single strands, isolated with streptavidin-coated magnetic particles (Dynabeads M-280) as described by Hultman et al. (13).

RESULTS

The samples from the healthy volunteers all produced distinct signals by interaction with the wild-type oligo-

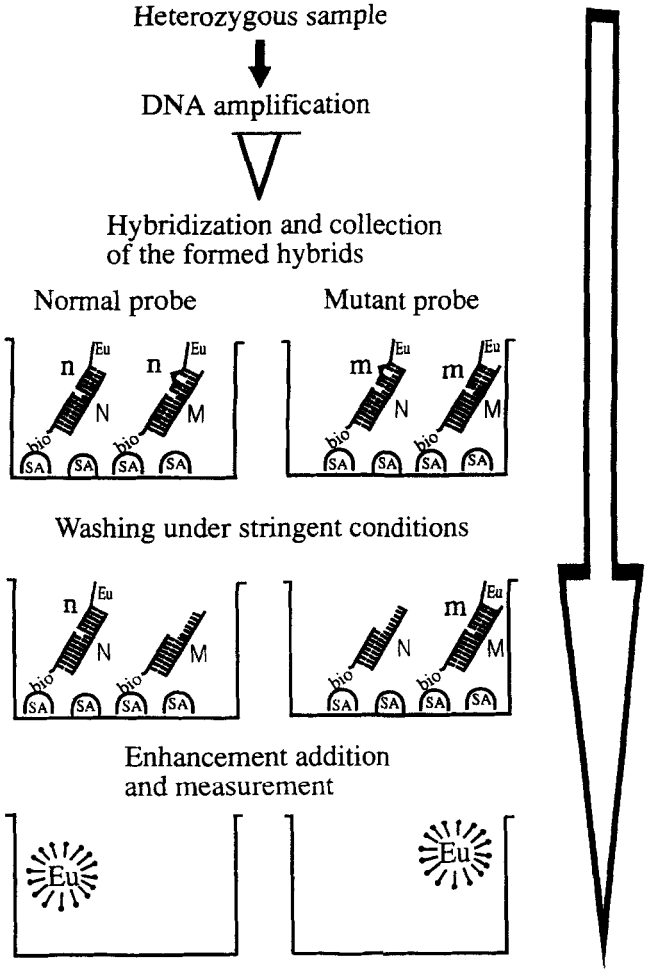

Fig. 1. Schematic outline of the analytic procedure carried out with a sample from an individual heterozygous for the apoB-3500 mutation. In this case a fluorescent signal is generated with both the wild type probe and the mutant probe. SA, streptavidin; bio, biotin label on the "universal" probe (WO 66); n, wild-type probe (WO 68); m, mutant probe (WO 69); N, wild-type PCR-product; M, mutant PCR-product.

nucleotide, the amplitude never being lower than 180,000 counts per second (cps) and varying up to 250,000 cps, while interaction with the mutant probe only generated between 5,000 to 10,000 cps. The latter figure was slightly higher when the rinsing procedure was carried out at a temperature lower than 35°C. With the control DNA from the patient with FDB, 110,000–130,000 cps were obtained with each of the two oligonucleotides (WO 68 and WO 69). The analyses were performed in duplicate. The coefficient of variation, calculated from the duplicate determinations, was less than 9%. In the blanks, without DNA, only 400–1200 cps were recorded.

Of the 127 patient samples screened, 125 showed reactions with the wild-type probe of the same magnitude as those from the healthy volunteers, whereas two specimens displayed a positive signal with both the wild-type and the mutant oligonucleotide. In the latter two samples, the number of counts using WO 68 and WO 69 were in the same range as those obtained using the DNA from the patient with FDB. This strongly suggested that these samples were heterozygous for the mutant gene. None of the other samples generated more than 5,000–10,000 cps with the mutant probe. The identity of the mutation (3) was confirmed by sequence analysis of the PCR products (data not shown).

The two patients were a male (A), aged 31 yr, and a female (B), aged 63 yr (age at sampling). The two patients were not, as far as we could find out, related to each other. Both patients had prominently elevated total serum and LDL-cholesterol (9.5 and 8.5 mmol/l respectively for patient A, and 8.5 and 6.3 mmol/l for patient B), but normal levels of serum triglycerides, HDL-cholesterol, and VLDL-cholesterol. The levels of apoB were also increased (1.93 g/l for patient A and 2.19 g/l for patient B) but not those of Lp[a]. Both patients were found to have apoE genotype 3/3. Clinically, both patients had tendon xanthomas, and one of them (B) also had bilateral arcus cornae. Patient B, who suffered from advanced obesity, had serious signs of cardiovascular disease with several myocardial infarctions and bypass surgery before her death from a myocardial infarction at 64 yr of age. So far, patient A has not shown any clinical evidence of atherosclerosis.

For both patients, a family history of lipid disturbances was found. The father of patient A had hypercholesterolemia and tendon xanthomas, which had led to the clinical diagnosis of heterozygous FH. The patient has two children, one of whom also has elevated serum cholesterol. One sister of patient B has hypercholesterolemia, and one daughter of the patient was found to have hypercholesterolemia and tendon xanthomas.

DISCUSSION

The use of time-resolved fluorescence with lanthanide chelates (14) in clinical laboratories has recently been introduced in immunoassays, where it can replace isotopes such as ^{125}I (Delfia®, Wallac Oy, Turku, Finland). The emitted fluorescence from europium chelates shows a large Stokes' shift (340 versus 613 mm) and a narrow emission peak, which enhances the monitoring. Lantanide chelates are thus very suitable as markers in bioassays when high sensitivity is needed. The labeling of DNA with europium chelates now allows this detection system to be used in molecular biology (15). Coupling of several europium chelates to the DNA-molecule further enhances the sensitivity. Sensitivities achieved are similar to those obtained with ^{32}P-labeled oligonucleotide probes and scintillation counting. The technique of measuring the fluorescence a fixed time after excitation reduces the nonspecific fluorescence, thereby enhancing the strength of the signal. Accordingly, the fluorescence intensity of our positive reactions was 20–30 times higher than that generated by the negative interactions.

The technique was simple to introduce in the laboratory and easy to run. As part of the handling can be automatized, large quantities of samples may be processed simultaneously; furthermore, the method has a more general potential, as a number of different point mutations can be detected by the use of a battery of various labeled oligonucleotides. This is facilitated by the fact that oligonucleotide probes labeled with different lanthanides can be detected in the same solution after a single hybridization reaction (16). In addition, the microtiter format gives the possibility of using a set of probes in different microtitration wells. Another advantage with the present technique is the omission of isotopes. Furthermore, europium-labeled oligonucleotides can be stored at least 12 months without losing their activity. It is, however, necessary to have access to a fluorometer equipped for time-resolved fluorescence, but today this equipment is standard in many clinical laboratories.

Apart from the present technique, different approaches have been used to diagnose FDB. Originally the apoB-3500 mutation was identified by allele-specific hybridization, which is dependent on the use of ^{32}P-labeled probes and not very suitable for large scale investigations (17). Another technique introduced for screening patient samples for point mutations is minisequencing (18). However, this approach is still dependent on isotopes (^{3}H), and the storage time for the reagents is shorter (3 months). A possible alternative is allele-specific PCR, which allows analysis of many samples without the use of isotopes (19–22).

The frequency of FDB in the present study (1.6% of the investigated patients) is in agreement with other studies, where the frequency rate has varied between 1 and 6% in different hypercholesterolemic materials (8, 23–25). In Scandinavia, the disease has been identified in Denmark (8), but a similar investigation in Finland failed to reveal any positive individuals among patients with hypercholesterolemia (26).

In summary, the present paper describes a novel application of a new technique for detection of point mutations in the human genome. Utilizing the technique, the occurrence of FDB among hypercholesterolemic patients in Sweden has been demonstrated. The present method should be most useful for further investigations concerning the distribution of apoB-3500 in the general population.

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