

Familial apolipoprotein E deficiency and type III hyperlipoproteinemia due to a premature stop codon in the apolipoprotein E gene

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Abstract A kindred with apolipoprotein E deficiency and a truncated lower molecular weight apoE mutant, designated apoE-3_{Washington}, has been identified. Gel electrophoresis demonstrated complete absence of the normal apoE isoproteins and the presence of a small quantity of a lower molecular weight apoE. Plasma apoE levels in the proband were approximately 4% of normal. This marked deficiency of apoE resulted in delayed uptake of chylomicron and very low density lipoprotein (VLDL) remnants by the liver, elevated plasma cholesterol levels, mild hypertriglyceridemia, and the development of type III hyperlipoproteinemia. Sequence analysis of the patient's apoE gene revealed a single nucleotide substitution of an A for a G, which converted amino acid 210 of the mature protein, tryptophan (TGG), to a premature chain termination codon (TAG), thus leading to the synthesis of a truncated E apolipoprotein of 209 amino acids with a molecular mass of 23.88 kDa. Northern blot analysis of differentiated monocyte-derived macrophages demonstrated a mutant mRNA indistinguishable in size from normal apoE mRNA. The nucleotide substitution also resulted in the formation of a new restriction site for Mae I. Using this enzyme we were able to establish that the proband is a homozygote and that her two offsprings are heterozygous for the ϵ -3_{Washington} allele. ■ These data demonstrate that the striking deficiency of apoE-3_{Washington} results in a moderate form of type III hyperlipoproteinemia. The clinical presentation also suggests a dispensable role of apoE in the nervous system and in immunoregulation.—Lohse, P., H. B. Brewer III, M. S. Meng, S. I. Skarlatos, J. C. LaRosa, and H. B. Brewer, Jr. Familial apolipoprotein E deficiency and type III hyperlipoproteinemia due to a premature stop codon in the apolipoprotein E gene. *J. Lipid Res.* 1992. 33: 1583–1590.

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Apolipoprotein E is a 299 amino acid glycoprotein of 34.2 kDa (1). It is a constituent of chylomicrons, VLDL, and HDL, and mediates the cellular uptake of these particles through an interaction with LDL (apoB,E) and putative apoE receptors (2–5).

ApoE is a genetically polymorphic plasma protein with

three common alleles that code for the isoproteins apoE-2, apoE-3, and apoE-4 (for review see ref. 6). The ϵ -3 allele has the highest frequency in the population and apoE-3 is therefore considered to be the parent isoprotein. Homozygosity for apoE-2(Arg₁₅₈→Cys) predisposes to the development of type III hyperlipoproteinemia presumably due to a reduced binding affinity of apoE-2 for the LDL receptor of less than 2% that of apoE-3 (7). This leads to a delayed metabolic clearance of apoE-containing particles in vivo. ApoE-4 (Cys₁₁₂→Arg) demonstrates normal binding to the LDL receptor in vitro (7), but is catabolized more rapidly when compared to apoE-3 as shown by in vivo kinetic studies (for review see refs. 8 and 9).

Type III hyperlipoproteinemia is characterized by elevated plasma cholesterol and triglyceride levels and the accumulation of cholesterol and apoE-enriched lipoprotein remnants of both intestinal and hepatic origin (broad- β or floating- β disease). Clinical features include tuberous as well as palmar xanthomas and the development of premature coronary and peripheral atherosclerosis (for review see refs. 8, 10–12).

A very rare form of type III hyperlipoproteinemia is due to apolipoprotein E deficiency. The only kindred described thus far had no detectable plasma apoE (13) and markedly decreased levels (14, 15) of two abnormal apoE mRNAs (14). Cloning of the apoE gene from the apoE-deficient patient and subsequent DNA sequence analysis revealed an A to G substitution in the acceptor splice site

Abbreviations: apo, apolipoprotein; bp, base pairs; HDL, high density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins.

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of the third intron of the apoE gene (16). This mutation caused aberrant splicing of the third intron, thus producing two apoE mRNAs containing either the entire or the 3'-portion of the third intron. Translation stop codons within these intronic sequences led to the production of apoE peptides of approximately 10,000 Da, which were not immunoprecipitable with a polyclonal apoE antibody (14, 16). The nucleotide substitution also created a new restriction site for the enzyme Sac II (16). Using a polymerase chain reaction product that contained the 3'-end of intron 3 and the 5'-end of exon 4 and the restriction fragment length polymorphism for Sac II, we were able to demonstrate that the proband is homozygous for this mutation (P. Lohse, unpublished data).

In the present report we describe a new kindred with apoE deficiency due to a G to A substitution in the apoE gene, which replaces amino acid 210, tryptophan (TGG), with a premature stop codon (TAG). This leads to the synthesis of a truncated apoE with a predicted molecular weight of 23,880 and is proposed to be the basis for the type III hyperlipoproteinemia observed in the homozygous patient.

MATERIALS AND METHODS

Clinical data

The proband is a 48-year-old white female who presented at age 43 with fasting plasma cholesterol and triglyceride levels of 358 and 135 mg/dl, respectively. VLDL cholesterol, LDL cholesterol, and HDL cholesterol were 140, 131, and 87 mg/dl, respectively. The diagnosis of type III hyperlipoproteinemia was established by a VLDL cholesterol to plasma triglycerides ratio of 1.04 (normal <0.3). The patient currently follows a low-fat diet without medication. Recent plasma cholesterol and triglyceride values were 291 and 121 mg/dl, respectively. VLDL cholesterol was 92 mg/dl; LDL cholesterol, 138 mg/dl; and HDL cholesterol, 61 mg/dl. The VLDL cholesterol to plasma triglycerides ratio remained elevated at 0.76.

Isolation and characterization of plasma lipoproteins

Na₂EDTA blood was collected after an overnight fast and plasma was obtained by low-speed centrifugation at 4°C. Total plasma cholesterol and triglyceride concentrations were measured with an Abbott VPSS analyzer (Abbott Lab., North Chicago, IL). HDL cholesterol was quantitated by dextran-sulfate precipitation of plasma (17). VLDL cholesterol and LDL cholesterol values were determined after sequential ultracentrifugation in a 40.3 Beckman Rotor (Beckman Instruments, Inc., Fullerton, CA; refs. 18, 19). ApoE was measured by radioimmunoassay as previously described (20).

Plasma fractions were dialyzed against 10 mM am-

monium bicarbonate and delipidated in chloroform-methanol 2:1 prior to gel electrophoresis. Protein concentrations were determined by the Bradford method (21).

Apolipoprotein gel electrophoresis

SDS polyacrylamide slab gel electrophoresis (5 or 15% acrylamide, 0.5% bisacrylamide, 0.1% SDS) was performed in a Bio-Rad 220 Dual Slab Cell (Bio-Rad Laboratories, Richmond, CA) for 3–4 h at 200 V and 4°C (22).

Two-dimensional analysis of plasma apolipoproteins was carried out by isoelectric focusing in the first dimension followed by SDS polyacrylamide gel electrophoresis in the second dimension as previously described (23). Immunoblotting (24) was performed using a monoclonal apoE antibody.

DNA isolation and amplification by the polymerase chain reaction

High molecular weight chromosomal DNA was isolated from white blood cells and amplified using apoE-specific primers synthesized by the phosphoramidite method (25). Amplification of exon 3 of the human apoE gene was performed in a reaction mixture of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, and 200 μM each dATP, dCTP, dGTP, and dTTP. The fourth exon was amplified in the aforementioned buffer supplemented with 10% (v/v) dimethyl sulfoxide. A typical 30-cycle profile included melting at 94°C for 1 min, annealing at 62°C for 1 min 20 sec, and polymerization at 72°C for 2 min.

DNA sequence analysis

Amplified DNA was subcloned into vectors M13mp18 and mp19 (New England BioLabs, Inc., Beverly, MA; ref. 26) and sequenced by the dideoxynucleotide chain termination method (27) with T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH).

Restriction fragment length polymorphism analysis

PCR-amplified DNA spanning exon 4 was isolated by 1.5% low melting point agarose (Bethesda Research Laboratories) gel electrophoresis and digested with 2.7 units of the restriction enzyme Mae I (Boehringer Mannheim, Indianapolis, IN) for 2 h at 45°C according to the manufacturer's instructions. The fragments were separated on a 1.5% agarose minigel at 25 mA for 3 h. DNA was stained with ethidium bromide.

Northern blot analysis of monocyte-derived macrophage RNA

Monocyte-derived macrophages from the patient and from a control subject were isolated and cultivated as previously described (28). Total RNA from differentiated macrophages was extracted by the guanidine isothiocyanate method (29) at days 7 and 21. Ten-μg samples of total RNA were electrophoresed on a 1% formaldehyde/

agarose gel (30) and transferred to a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH). The blot was hybridized with a uniformly ^{32}P -labeled full-length apoE cDNA probe (15), washed as outlined in the protocol of the manufacturer, and exposed to X-ray film.

RESULTS

Apolipoprotein gel electrophoresis

Two-dimensional gel electrophoresis demonstrated the complete absence of the normal apoE isoproteins, at their typical positions, in the patient's plasma, when compared to that of plasma from a normal subject (data not shown). Quantitation of apoE by radioimmunoassay revealed that the apoE level was markedly decreased to 0.15 mg/dl, which is approximately 4% of normal values (3.5 ± 1 mg/dl).

The increase in concentration of lipoprotein remnants (β -VLDL) in the plasma was confirmed by 5% SDS polyacrylamide gel electrophoresis comparing chylomicrons/VLDL and LDL of the patient with chylomicrons/VLDL and LDL of a normal subject. As illustrated in **Fig. 1**, apoB-48, a marker apolipoprotein for lipoproteins of intestinal origin, was significantly elevated in the chylomicron/VLDL fraction and especially in the LDL of the apoE-deficient patient. This is consistent with a defect in liver uptake of remnant lipoproteins due to the low levels of plasma apoE.

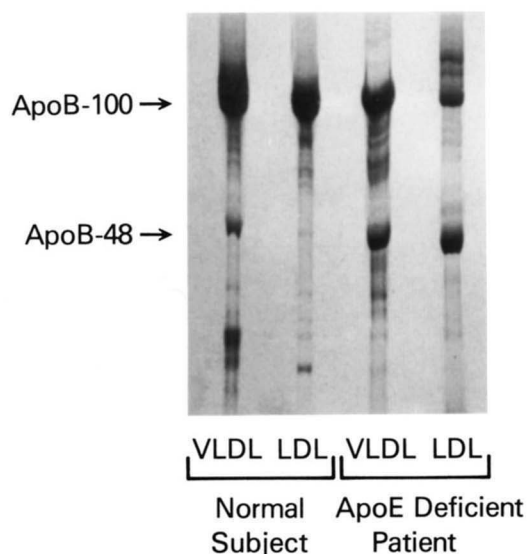


Fig. 1. One-dimensional SDS polyacrylamide gel electrophoresis of 150 μg each apoVLDL and apoLDL from a normal subject and from the apoE-deficient patient. Chloroform-methanol-delipidated VLDL and LDL from a control subject and from the apoE-deficient proband were separated on a 5% SDS polyacrylamide gel. After fixation of the gel, proteins were visualized by staining with Coomassie Brilliant Blue R-250. The positions of apoB-100, a marker for lipoproteins of hepatic origin, and of apoB-48, a protein component of intestinally derived chylomicrons, are shown on the left side.

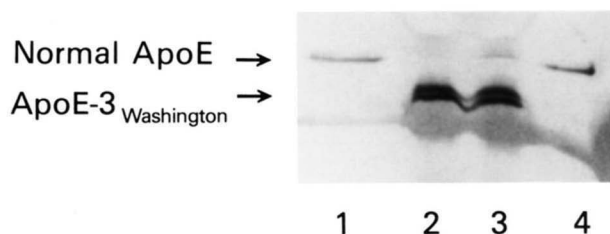


Fig. 2. Immunoblot of normal apoE-3 and apoVLDL from the apoE-deficient patient (enlargement). ApoE-3 standard (lanes 1 and 4), 400 μg of the patient's apoVLDL (lane 2), and 400 μg of the patient's apoVLDL mixed with apoE-3 standard (lane 3) were separated on a 15% SDS polyacrylamide gel, transferred to nitrocellulose and incubated with a monoclonal apoE antibody. The positions of both normal apoE-3 and apoE-3_{Washington} (riding on top of apoA-I) are indicated on the left side.

To further evaluate the small quantity of apoE present in the apoE-deficient patient, we determined the apoE content of the lipoprotein fractions. The proband's VLDL, LDL, HDL, and 1.21 g/ml infranate contained 0.05, 0.01, 0.02, and 0.04 mg/dl apoE, respectively. However, two-dimensional gel electrophoresis of the VLDL, LDL, and HDL fractions followed by immunoblotting failed to visualize any apoE.

To facilitate the detection of apoE in the proband, a Western blot of 400 μg of the patient's apoVLDL was performed after separation by one-dimensional 15% SDS polyacrylamide gel electrophoresis. **Fig. 2** illustrates an enlargement of the original immunoblot. The apoE present in the proband had a significantly lower molecular weight than purified apoE-3 from a normal subject and was estimated to have a mass of approximately 25 kDa.

DNA sequence analysis

Genomic DNAs from a normal subject and from the apoE-deficient patient were amplified by the polymerase chain reaction using apoE-specific primers. Sequence analysis of the apolipoprotein coding exons as well as their donor and acceptor splice sites in both subjects led to the identification of a single G to A substitution at nucleotide 447 in the fourth exon of the proband's apoE gene which results in the introduction of a premature translational stop codon TAG (**Fig. 3**). This mutation occurs at a position corresponding to amino acid 210, tryptophan (TGG), of the mature apoE, thus leading to the synthesis of a truncated 209 residue protein with a calculated molecular mass of 23,880 Da (theoretical pI 5.35 instead of 5.32 for apoE-3). The parent genetic allele was ϵ -3 and the apoE mutant was therefore designated apoE-3_{Washington}.

Polymorphic restriction enzyme sites

Computer analysis (PC/GENE; IntelliGenetics, Mountain View, CA) of the ϵ -3_{Washington} allele revealed that the mutation created a new restriction site for the enzyme

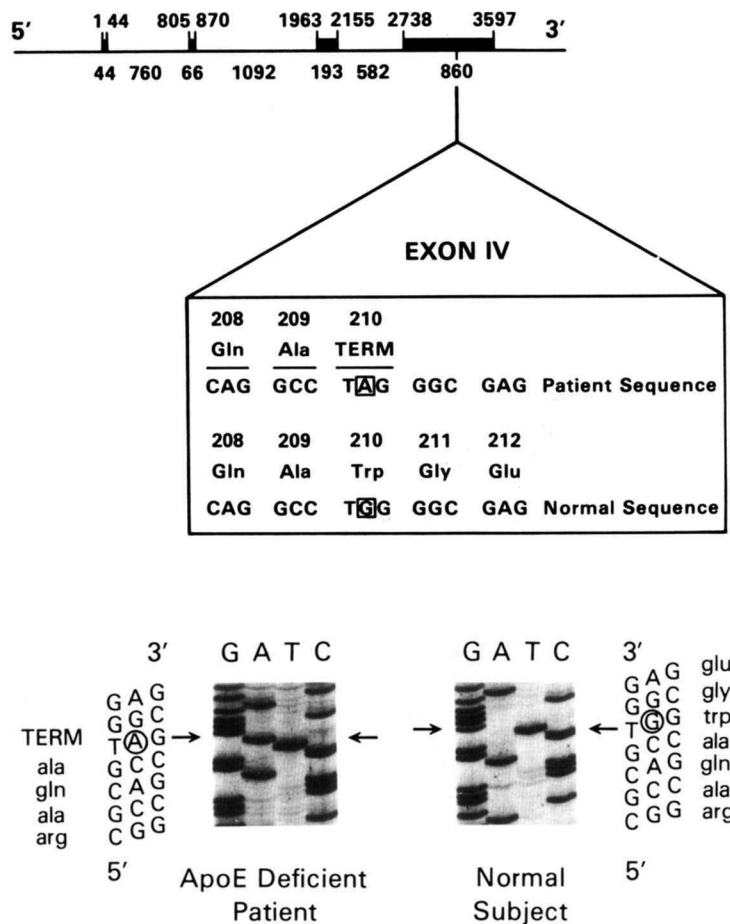


Fig. 3. Representation of the human apoE gene and the mutation identified in the patient with apoE deficiency. A schematic diagram of the genomic structure of apoE (upper panel) illustrates the location of the mutation in the fourth exon of the apoE-3^{Washington} gene. A nucleotide substitution of an A for a G replaces tryptophan (TGG), amino acid 210 of the mature apoE, with a premature termination codon (TAG). The corresponding autoradiogram of a sequencing gel of DNA from a normal subject with the phenotype apoE-3/3 (right lanes) and from the apoE-deficient patient (left lanes) is shown in the lower panel. The position of the mutation is indicated by the arrows.

Mae I (C/TAG). Digestion of DNA amplified by the polymerase chain reaction using primers 3 and 6 (25) allowed us to identify the mutation in members of the Washington kindred. **Fig. 4** illustrates the electrophoretic analysis of amplified and Mae I-digested DNA from a control subject, the proband, and her two offsprings. The restriction enzyme Mae I is expected to cleave the patient's 785 bp amplification product once, leading to the formation of two abnormally sized fragments of 505 and 280 bp in length. In the proband, only these two smaller fragments were visible, confirming that she is a homozygote for the mutation. Her daughter and son, however, had both the normal, uncleaved PCR product as well as the two abnormal restriction fragments, establishing them as being heterozygous for the G to A substitution.

Northern blot analysis of monocyte-derived macrophage RNA

Monocyte-macrophages isolated from the proband and cultivated in vitro for 7 and 21 days contained apoE mRNA of about the same size as found in control macrophages (data not shown).

DISCUSSION

Apolipoprotein E is essential for the normal catabolism of intestinally derived chylomicron remnants and hepatically derived VLDL remnants via LDL (apoB,E) and putative apoE receptor pathways. It is also necessary for

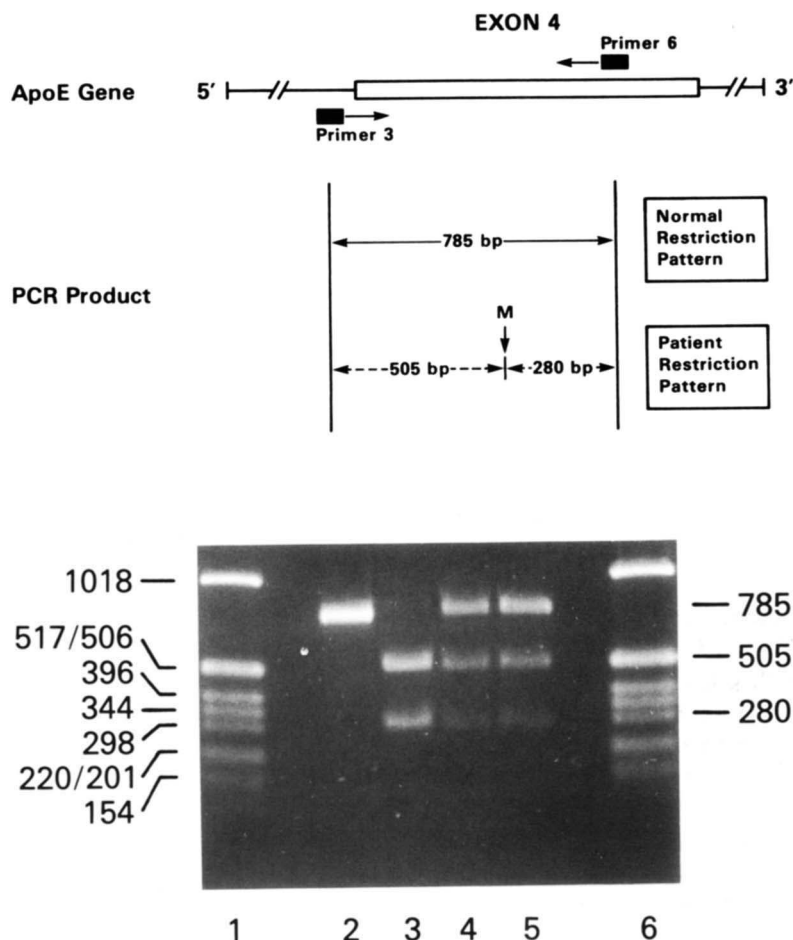


Fig. 4. Restriction enzyme digestion of PCR-amplified DNA from members of the Washington kindred and from a control subject. Primers 3 and 6 (25) were used to amplify the region of the mutation within exon 4 of the human apoE gene as shown in the upper panel. The resulting product of 785 bp was then digested with the restriction enzyme Mae I, which cuts at the site indicated by the letter M in the patient's DNA and leads to the formation of two fragments of 505 and 280 bp in length. The lower panel illustrates the electrophoretic analysis of PCR-amplified DNA from a control subject (lane 2), the proband (lane 3), her son (lane 4), and her daughter (lane 5) after restriction enzyme digestion with Mae I. A nucleic acid molecular size standard is shown in lanes 1 and 6 and the size of the fragments is given on the left side. The sizes of the uncut and digested fragments are illustrated on the right side.

efficient conversion of VLDL to IDL and finally to LDL. A number of structural variants of apoE as well as apoE deficiency have been shown to result in an accumulation of cholesterol-enriched remnant particles (β -VLDL), type III hyperlipoproteinemia, and premature atherosclerosis (6, 8-12).

ApoE deficiency is an inherited genetic disease that had been previously described only in a single kindred (13-16). The proband in this earlier study had no immunodetectable apoE and her monocyte-derived macrophages synthesized only 1-3% of normal amounts of two aberrant apoE mRNAs. Sequence analysis revealed a single A to G substitution in the acceptor splice site of the third intron of the apoE gene.

In the present study we have analyzed a second patient with apoE deficiency who synthesizes a truncated form of plasma apoE. Physical examination was surprisingly unremarkable with no xanthomas or corneal arcus. There was, however, a strong history of hyperlipidemia and premature atherosclerosis in the family. Two paternal uncles had died in their 60s of myocardial infarction. A maternal uncle (age 66) and an aunt (age 63) as well as a sister (age 50) and a brother (age 46) of the patient have hypercholesterolemia.

The proband's lipid and lipoprotein cholesterol profile was similar to that of the first described patient with apoE deficiency and clearly differs from the values found in other subjects with type III hyperlipoproteinemia (13).

Plasma triglyceride levels were lower and the ratio of VLDL cholesterol to plasma triglycerides was higher than in the typical type III hyperlipoproteinemic patient. The only slightly elevated plasma triglyceride concentrations and the increased HDL cholesterol values may be due, however, in part to the fact that the proband has been on estrogen replacement therapy for more than 10 years (31, 32).

Two-dimensional electrophoresis of plasma followed by protein staining demonstrated that the normal apoE iso-protein pattern was completely absent in this patient. Two-dimensional electrophoretograms of the patient's VLDL, LDL, and HDL fractions and immunoblotting with a monoclonal apoE antibody also failed to detect any apoE. Quantitation by radioimmunoassay, however, revealed very low concentrations of apoE in plasma (approximately 4% of normal values), the lipoprotein fractions, and the infranate. The failure to visualize apoE was therefore interpreted as being due to the inability to load sufficient protein on the isoelectric focusing gel.

One-dimensional 5% SDS polyacrylamide gel electrophoresis of 150 μ g each of the proband's apoVLDL and apoLDL demonstrated an increased concentration of apoB-48, a marker for lipoproteins of intestinal origin, in both density fractions, especially pronounced in LDL (Fig. 1). These results are in agreement with previous observations in the first reported kindred with apoE deficiency (13, 33, 34) and illustrate the essential role of apoE for receptor-mediated removal of remnants of chylomicrons and VLDL. A protein load of 400 μ g apoVLDL was necessary to detect apoE by immunostaining. An enlarged portion of this Western blot is shown in Fig. 2, demonstrating the presence of a significantly smaller apoE of approximately 25 kDa in the apoE-deficient patient.

To identify the underlying molecular defect in this subject, portions of the apoE gene of the apoE-deficient patient and of a normal subject were amplified by the polymerase chain reaction. Sequence analysis of the protein coding regions of the apoE gene of the apoE-deficient patient revealed a single nucleotide substitution at position 447 of the fourth exon (Fig. 3) which replaced amino acid 210, tryptophan (TGG), with a premature translational stop codon (TAG) and resulted in a truncated apoE with 209 instead of 299 amino acids and a predicted molecular weight of 23,880 instead of 34,236. The size of the mutant apoE mRNA was indistinguishable from that found in control macrophages, consistent with a point mutation in the patient's apoE gene.

The mutation also created a new restriction site for the enzyme Mae I (C/TAG). Digestion of PCR-amplified DNA spanning the region of the G to A substitution with this enzyme established that the proband is a homozygote (Fig. 4), although no consanguinity could be documented. However, we cannot rule out a large deletion of one apoE

allele. Southern blot hybridization, similar amounts of PCR-generated products, and analysis of the patient's two offsprings render this highly unlikely. The son (age 28) and daughter (age 21) are both heterozygotes for the mutation as demonstrated by the restriction fragment length polymorphism analysis (Fig. 4), and they have plasma apoE levels of less than 50% of normal. Plasma lipid and lipoprotein cholesterol values of the two offsprings were found to be within the normal range.

The truncated E apolipoprotein probably has retained its full activity to bind to the LDL receptor, based on previous observations with the amino-terminal 22 kDa thrombolytic fragment (residues 1-191; ref. 35). The mutant also contains the attachment site for carbohydrate side chains, which has been localized at amino acid 194, threonine (36). This may explain the unusual double band seen on the immunoblot of the proband's apoVLDL (Fig. 2) which suggests that approximately 50% of apoE-3_{Washington} is glycosylated. ApoE-3_{Washington} lacks, on the other hand, the complete carboxyl-terminal third which contains four amphiphilic α -helices (residues 203-221, 226-243, 245-266, and 268-285) proposed to play an important role in lipid binding (1, 37). However, the mutant protein has at least limited ability to bind to lipoprotein particles, since we were able to visualize apoE-3_{Washington} in the patient's apoVLDL. This is in agreement with earlier reports demonstrating binding of the 22 kDa thrombolytic fragment and of various cyanogen bromide peptides of apoE to dimyristoylphosphatidylcholine (35, 38). Furthermore, it would be anticipated that apoE-3_{Washington} would not form tetrameric complexes in aqueous solutions by self-association (39), since this property has been attributed to the carboxyl-terminal region of apoE (amino acids 225-299) and could be modeled by the 10 kDa thrombolytic peptide (residues 216-299). The tendency of the amino-terminal fragment to self-associate, in contrast, was low and the 22 kDa thrombolytic peptide was found to be a predominantly monomeric protein of roughly globular shape (40, 41).

Therefore, it may be speculated that apoE-3_{Washington}, although resembling a globular protein, is metabolically less stable than apoE-3 and/or has reduced ability for lipid binding, which could explain the low plasma apoE levels found in this apoE-deficient patient. Still, we cannot rule out the possibility that the mutant protein is catabolized mainly intracellularly due to an impaired cytoplasmic transport, as has been reported for the Lebanese nonsense mutation of the LDL receptor (42).

In summary, apoE deficiency due to a truncated lower molecular weight apoE mutant, designated apoE-3_{Washington}, results in type III hyperlipoproteinemia and premature cardiovascular disease. The disturbances of lipoprotein metabolism created by the low levels of apoE-3_{Washington} appear to be less pronounced than those caused by structural variants of apoE associated with impaired binding

to the LDL (apoB,E) receptor. The lack of clinical symptoms related to the nervous and immune systems in apoE deficiency suggests that additional proposed functions of apoE can either be assumed by other proteins or are of minimal physiological importance. ■

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