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Research Article

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A Novel Electrophoretic Variant of Human Apolipoprotein E

Identification and Characterization of Apolipoprotein E1

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Abstract. A new apolipoprotein E (apo E) phenotype has been demonstrated in a Finnish hypertriglyceridemic subject (R.M.). At the time of this study, R.M.'s plasma triglyceride and cholesterol levels were 1,021 and 230 mg/dl, respectively. The subject's apo E isoelectric focusing pattern was characterized by two major bands, one in the E3 position and the other in the E1 position. Normally the E1 position is occupied by sialylated derivatives of apo E4, E3, or E2. The E1 band of subject R.M. is not a sialylated form, however, because it was not affected by neuraminidase digestion. The identity of the E1 variant as a genetically determined structure was established by amino acid and partial sequence analyses, confirming that the variant is an example of a previously uncharacterized apo E phenotype, E3/1. Both cysteamine modification and amino acid analysis demonstrated that this variant contains two cysteine residues per mole. Sequence analysis of two cyanogen bromide fragments and one tryptic fragment of the apo E3/1 showed that it differs from E2(Arg_{158} → Cys) at residue 127, where an aspartic acid residue is substituted for glycine. This single amino acid interchange is sufficient to account for the one-charge difference observed on isoelectric focusing gels between E2(Arg_{158} → Cys) and the E1 variant. The variant has been designated E1(Gly_{127} → Asp, Arg_{158} → Cys). When compared with apo E3, the E1 variant demonstrated reduced ability to compete with ^{125}I-LDL for binding to LDL (apo B,E) receptors on cultured fibroblasts (~4% of the amount of binding of apo E3). This defective binding is similar to that of E2(Arg_{158} → Cys). Therefore, the binding defect of the variant is probably due to the presence of cysteine at residue 158, rather than aspartic acid at residue 127. In contrast, the apo E3 isoform from this subject demonstrated normal binding activity, indicating that it has a normal structure. In family studies, the vertical transmission of the apo E1 variant has been established. It is not yet clear, however, if the hypertriglyceridemia observed in the proband is associated with the presence of the E1(Gly_{127} → Asp, Arg_{158} → Cys) variant.

Introduction

Apolipoprotein E (apo E)[1] exhibits polymorphism that is genetically determined (1–4), with the genetic influence expressed at the level of the apo E structural gene (5, 6). On isoelectric focusing gels, three major isoforms of apo E, designated E4, E3, and E2, have been demonstrated (7). The presence of minor, more acidic isoforms arises from variable sialylation of the major isoforms (3). At present, five structural forms of the protein have been described (5, 6, 8, 9). Compared with apo E3 (the parent form of the apo E molecule), the variants differ in primary structure involving single amino acid substitutions. The variants are designated on the basis of their position on isoelectric focusing

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1. *Abbreviations used in this paper:* apo B, apo C, and apo E, apolipoproteins B, C, and E, respectively; DMPC, dimyristoylphosphatidylcholine; SDS, sodium dodecyl sulfate.

gels and their amino acid differences compared with apo E3. They are referred to as E4($Cys_{112} \rightarrow Arg$), E2($Arg_{145} \rightarrow Cys$), E2($Lys_{146} \rightarrow Gln$), and E2($Arg_{158} \rightarrow Cys$).

According to the three allele genetic model for apo E as originally proposed by Zannis and Breslow (3, 4), each allele determines one of the isoelectric variants (E2, E3, or E4). Thus, six phenotypes were predicted: three homozygous (E2/2, E3/3, and E4/4) and three heterozygous (E4/3, E4/2, and E3/2). The demonstration that the E2 phenotype represents different primary structures, E2($Arg_{158} \rightarrow Cys$), E2($Arg_{145} \rightarrow Cys$), and E2($Lys_{146} \rightarrow Gln$), required modification of this model to include two additional E2 alleles for apo E (8, 9). In spite of this additional heterogeneity within the E2/2 phenotype, the results from several phenotyping studies carried out in various laboratories have indicated that all screened individuals could still be categorized by one of the six phenotypes (3, 10–14).

Apolipoprotein E is a component of several classes of plasma lipoproteins (15, 16). Because of its ability to bind to lipoprotein receptors on cells, which results in the internalization and degradation of the lipoprotein particles, apo E plays a central role in plasma lipid metabolism (for review see references 15 and 16).

The lipid disorder familial dysbetalipoproteinemia underscores the role of apo E in lipid metabolism. This disorder, either with (type III hyperlipoproteinemia) or without elevated plasma lipid levels, is associated with the presence of apo E2 (17–21). In most instances, affected subjects are homozygous for E2, but there are examples of subjects having other phenotypes (9, 22, 23). Type III hyperlipoproteinemia is characterized by the accumulation of β-very low density lipoproteins (β-VLDL) in the plasma (for review see reference 21). These cholesterol-rich lipoproteins represent chylomicron remnants, containing primarily apo B-48 and apo E, and hepatic VLDL, containing apo B-100 and apo E (24). The observation that the three E2 variants demonstrate reduced ability to bind to apo B,E (low density lipoproteins, LDL) receptors of fibroblasts has led us to postulate that the receptor binding dysfunction in these variants is a necessary factor for the expression of the type III disease (15, 16, 21).

In the present study, we have described an apo E phenotype that represents a variation of the six commonly recognized phenotypes. This phenotype is characterized by the presence of a genetically determined apo E structure that focuses in the E1 position, a position usually occupied only by sialylated derivatives. Recently, Gregg et al. (25) observed an apo E variant that focused in the E1 position on isoelectric focusing gels, and they described two phenotypes that appear to correspond to E3/1 and E2/1.

Methods

Plasma lipoprotein isolation and characterization. Plasma (EDTA) was obtained from subject R.M. after an overnight fast, and the lipoproteins were isolated by sequential ultracentrifugation using KBr to adjust the density (26). Triglyceride and total cholesterol were determined enzymatically (Bio-Dynamics, Boehringer Mannheim Corp., Indianapolis, IN). Phospholipid was determined by phosphorous analysis (27), and protein was determined by the method of Lowry et al. (28). Plasma apo E levels were determined by radioimmunoassay essentially as described (29). Paper electrophoresis was performed as previously described (30).

The $d < 1.006$ lipoproteins were subfractionated at 4°C on a 6% agarose column (Biogel A-5m, Bio-Rad Laboratories, Richmond, CA) (2.5 × 100 cm), which was equilibrated with buffer containing 0.15 M NaCl and 10 mM sodium phosphate (pH 7.4). The lipoproteins (21 mg of lipoprotein protein) were applied in a sucrose solution (20% w/v final concentration), and the column was eluted at a flow rate of 15 ml/h with the use of a peristaltic pump. The absorbance at 280 mm was monitored, and 15-min fractions were collected. Electron micrographs of negatively stained preparations (31) were obtained on a JEOL model JEM 100CX II electron microscope (JEOL USA Electron Optics, Peabody, MA), and the diameters of lipoprotein particles were measured from photomicrographs enlarged 100,000 times.

Apo E isolation and characterization. The $d < 1.006$ lipoproteins were delipidated with $CHCl_3$/MeOH (2:1), and the apoprotein mixture was solubilized in 6 M guanidine containing 0.1 M Tris and 0.01% EDTA (pH 7.4), as previously described (5). Apo E was separated from the apoprotein mixture by gel filtration on Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) in a glass column (2.5 × 300 cm, Kontes, San Leandro, CA) equilibrated with 4 M guanidine containing 0.1 M Tris, 0.1% β-mercaptoethanol, and 0.01% EDTA (pH 7.4). Pooled fractions were exhaustively dialyzed against 5 mM NH_4HCO_3 and lyophilized. Purity was assessed by sodium dodecyl sulfate (SDS) gel electrophoresis (32).

Analytical isoelectric focusing and cysteamine modification of VLDL were performed as previously described (5). Preparative isoelectric focusing was performed on a LKB multiphor flatbed unit (LKB Produkter, Bromma, Sweden) over a pH range of 4–6.5 (2% Pharmalyte, Pharmacia Fine Chemicals) at 4°C as described (5). The paper print technique was used to locate the isoform bands and proteins were eluted from the Sephadex G-200 support medium with 4 M guanidine containing 0.1 M Tris and 0.01% EDTA (pH 7.4). Ampholytes were removed from the protein by dialysis and $(NH_4)_2SO_4$ precipitation as described (5). With this procedure, the apo E3 and apo E1 were isolated and purified for further characterization.

Two-dimensional gel electrophoresis on the isolated apo E was carried out using a 0.75-mm-thick polyacrylamide slab for the isoelectric focusing in the first dimension (Ampholine, pH 4–6; LKB Produkter). A section of the gel corresponding to one lane was cut from the slab and incubated with 0.02 M Tris containing 1% SDS, 10% glycerol, and 1% β-mercaptoethanol (pH 8.2). The second dimension, representing a 12.5% acrylamide gel, was run as described (5). Digestions of apo E3/1 with neuraminidase (Sigma Chemical Co., St. Louis, MO) were performed at 37°C for 4 h in 0.1 M NH_4OAc (pH 4.0) at a substrate to enzyme ratio of 20:1.

Amino acid and sequence analyses. Samples for amino acid analysis were hydrolyzed for 20 h at 110°C in 6 N HCl in evacuated, sealed hydrolysis tubes. The dried hydrolysate was dissolved in 0.2 N sodium citrate (pH 2.2) and analyzed on a Beckman 121MB analyzer equipped with a model 126 Data System (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). No correction was made for the hydrolytic destruction of serine or threonine.

The cyanogen bromide digestion was carried out with a 30-fold excess (w/w) of CNBr (Pierce Chemical Co., Rockford, IL), and the CB4 and CB5 fragments were isolated as previously described (5, 6). The tryptic digestion of isolated and purified apo E1 was carried out at

a substrate to enzyme ratio of 25:1 for 3 h at 37°C. The tryptic peptides were isolated by high voltage paper electrophoresis at pH 1.9 and pH 6.4 as previously described (6).

Peptides for sequence analysis were dissolved in 0.5 ml 50% acetic acid and degraded in the presence of 2 mg polybrene (Sigma Chemical Co.) on a Beckman 890C sequencer (Beckman Instruments, Inc.) using a 0.1 M Quadrol program (No. 122974). The phenylthiohydantoin amino acids were identified and quantitated by high performance liquid chromatography as described (5).

Fibroblast binding studies. Phospholipid complexes of the various apo E preparations were prepared by incubating 150 μg of protein (0.5–1.0 mg/ml in 0.1 M NH_4HCO_3, containing 0.1% β-mercaptoethanol) with sonicated dimyristoylphosphatidylcholine (DMPC) vesicles at a protein to lipid ratio of 1:4. The mixture was incubated at 25°C for 1 h, and the complexes were isolated by density gradient centrifugation (33). The ability of the various apo E complexes to compete with ^{125}I-LDL for binding to apo B,E receptors on cultured human fibroblasts was determined as previously described (33).

Results

Characterization of the proband. The proband, R.M., a native and inhabitant of Finland, has had a 16-yr history of hypertriglyceridemia and hypercholesterolemia (range: cholesterol, 225–550 mg/dl; triglyceride, 50–1,000 mg/dl). During this time, he has been followed by physicians in the Third Department of Medicine at the University of Helsinki, Finland. The common secondary causes of lipid abnormalities have been excluded. Lipase assays revealed that both the lipoprotein lipase and hepatic lipase values were within the normal range, and there was no obvious deficiency of apo C-II (Ehnholm, C., and T. Kuusi, unpublished observations). He has received various forms of medication without complete normalization of the hyperlipidemia. However, the hyperlipidemia has been most responsive to dietary restriction of calories and restriction of fat and cholesterol intake. The motivation of the subject has been variable over the years. Clinically, the subject was described as being mildly obese (182 cm; 85 kg), with a history of periodic exacerbation of hypertriglyceridemia and hypercholesterolemia accompanied by eruptive xanthomas. The xanthomas, present on the elbows, wrists, and knees, have come and gone over the 16-yr period. Palmar xanthomas were never described. The subject is asymptomatic of cardiovascular disease; however, inverted T waves have appeared on his electrocardiograms, first noted in 1980.

The lipoprotein phenotype has, at various times, resembled type III, IV, or V hyperlipoproteinemia. At the time of the present study, the hyperlipidemia of R.M. (49 yr of age in 1982) was difficult to categorize. His plasma triglyceride and cholesterol levels were 1,021 and 230 mg/dl, respectively. At that time he was receiving no medication. A distinctive feature of the lipid abnormality was the presence of the apo E phenotype E3/1 (to be described below) accompanied by a threefold elevation of the plasma apo E level (16 mg/dl, Table I). The hyperlipoproteinemia was characterized by gross elevations in the $d < 1.006$ fraction, with this fraction accounting for 92% of the plasma triglycerides, 80% of the cholesterol, and 81% of the apo E. The ratio of the $d < 1.006$ cholesterol to plasma triglycerides was 0.16. The subject had very low concentrations of LDL ($d = 1.02$–1.063) and high density lipoproteins (HDL) ($d = 1.063$–1.21); both fell below the fifth percentile levels for age- and sex-matched subjects (Table II) (34). An interesting feature of the LDL and HDL was their abnormally high triglyceride content: 32.4 and 12.9%, respectively. The composition and distribution of the lipoproteins of subject R.M. are shown in Table II.

The $d < 1.006$ lipoproteins were further characterized. Paper electrophoresis of the $d < 1.006$ fraction revealed lipoproteins extending from the pre-β into the β region (Fig. 1), suggesting that β-VLDL were present. Polyacrylamide gel electrophoresis on 4% gels demonstrated that the $d < 1.006$ lipoproteins from fasted plasma contained both the high molecular weight form (B-100) and the low molecular weight form (B-48) of apo B (Fig. 1). It has been shown that the presence of apo B-48 is indicative of chylomicrons or chylomicron remnants of intestinal origin (24, 35). Agarose chromatography of the $d < 1.006$ fraction resolved the $d < 1.006$ lipoproteins into two major fractions (Fig. 2 inset, *A* and *B*). The major fraction eluted in the void volume of the column. These lipoproteins remained at the origin on paper electrophoretograms (Fig. 2, inset) and ranged in size from 700–2,000 Å in diameter, as determined by negative staining electron microscopy. The major apoproteins were B-48 and apo E. This fraction resembled the chylomicron remnants sim-

Table I

Subject	Age	Sex	Ht	Wt	Apo E Phenotype	Plasma levels		
						Apo E*	Triglyceride	Cholesterol
			cm	*kg*				
R.M.	49	M	182	85	E3/1	16.0	1,021	230
Te.M.	13	M	160	49.6	E3/1	8.7	59	151
Tu.M.	12	M	151	49.8	E3/3	7.1	73	200
Ta.M.	8	F	129	29.5	—	5.5	49	216

* Apo E levels were determined by radioimmunoassay. Laboratory normal values: mean, 5.0 mg/dl; range, 3.5–7.0 mg/dl.

Table II. Plasma Lipid Concentrations and Distribution in Subject R.M.

Density fraction	Triglyceride*	Total cholesterol*	Phospholipid	Protein
	mg/dl	*mg/dl*	*mg/dl*	*mg/dl*
Plasma	1,021	230	—	—
$d < 1.006$	793.6	158.8	221.2	97.0
	(62.5)‡	(12.5)	(17.4)	(7.6)
$d = 1.006–1.02$	10.0	3.4	6.9	5.0
	(39.5)	(13.6)	(27.2)	(19.7)
$d = 1.02–1.063$	38.2	22.4	28.4	29.0
	(32.4)	(19.0)	(24.1)	(24.6)
$d = 1.063–1.21$	20.9	13.9	44.3	82.4
	(12.9)	(8.6)	(27.4)	(51.0)
	% distribution	*% distribution*	*% distribution*	*% distribution*
$d < 1.006$	92.0	80.0	73.5	45.5
$d = 1.006–1.02$	1.2	1.7	2.3	2.3
$d = 1.02–1.063$	4.4	11.3	9.5	13.6
$d = 1.063–1.21$	2.4	7.0	14.7	38.6

* Percent recovery of triglyceride and cholesterol among the lipoprotein fractions was 84.5 and 86.3%, respectively. Cholesteryl esters represented 69.3% of the plasma cholesterol. ‡ The percent composition is given in parentheses.

ilarly isolated from the fasting plasma of cholesterol-fed dogs and patients with type III hyperlipoproteinemia (24). The $d < 1.006$ fraction of R.M., which eluted from the column later (B), demonstrated β- and pre-β-electrophoretic mobility (Fig. 2 inset), and included apo B-100 and apo E as major protein constituents. The particles ranged in size from 300 to 900 Å in diameter. The features of this hyperlipidemia prevent classification of this subject as a typical type III hyperlipoproteinemic; however, it appears that the proband has a defect in clearing chylomicron remnants and processing VLDL into LDL. More detailed metabolic studies are required to characterize the hyperlipemia more completely.

Family studies were also conducted. Three of the four children of the proband were available for examination and were found to have normal triglyceride and cholesterol levels (Table I). Subject Te.M. was found to possess the E3/1 phenotype, thereby establishing vertical transmission of the E1 variant.

Characterization of the E1 variant. Isoelectric focusing of the VLDL from subject R.M. revealed two major isoform bands in the apo E region of the gel (Fig. 3). The presence of two major E isoforms is characteristic of heterozygous phenotypes; however, in this case only one of the bands, E3, focused in one of the three normally observed positions for apo E. The second major band focused in the E1 region, a position in which only minor sialylated isoforms are normally observed.

Previously, we have shown that the number of cysteine residues present distinguishes one major apo E isoform from another (5). To determine the number of cysteine residues in an isoform, the apo E cysteine residues are modified with cysteamine, a reagent that adds a positive charge to each cysteine residue (5). After cysteamine modification, the R.M. apo E isoform pattern shifted toward the cathode and resembled the E4/3 phenotype (Fig. 3). As will be substantiated below, the E3 band shifted one charge position, indicating the presence of one cysteine residue, and the E1 band shifted two charge positions, indicating the presence of two residues of cysteine.

To investigate the possibility that the E1 isoform represented a sialylated derivative of apo E, the apo E was isolated from the VLDL of the subject by gel filtration on Sephacryl S-300 and examined by two-dimensional gel electrophoresis. Both the E3 and E1 isoforms migrated with identical molecular weights (~34,000) (Fig. 4 *A*). Several minor, more acidic sialylated isoforms migrated with a slightly higher apparent molecular weight (designated as $E3_s$ and $E1_s$ in Fig. 4). Treatment of the isolated apo E with neuraminidase resulted in the disappearance of the sialylated forms but had no effect on the E1 band (Fig. 4 *B*). Cysteamine treatment of either a control or a neuraminidase-digested sample resulted in a shift of the pattern, again with no effect on the apparent molecular weight of the E1 (data not shown). We have designated this new form of apo E "E1" to indicate its focusing position on isoelectric focusing gels. This designation is consistent with the conventional nomenclature now agreed upon and used by most investigators (7).

The apo E3 and E1 isoforms were isolated by preparative

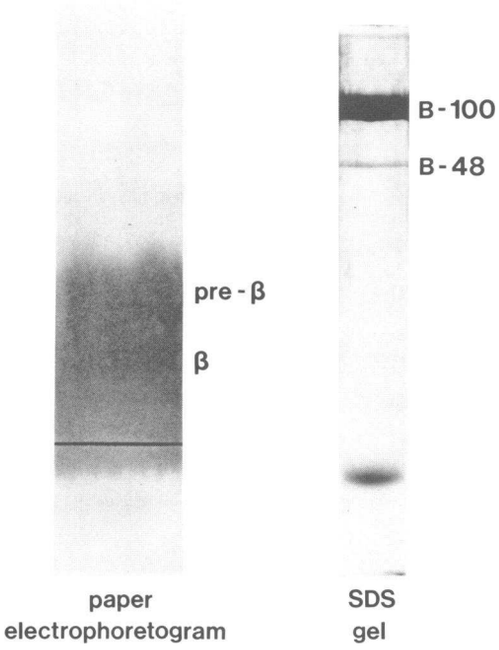

Figure 1. Paper electrophoretogram and 4% SDS-polyacrylamide gel of R.M.'s $d < 1.006$ lipoproteins. Approximately 150 μg of lipoprotein protein were applied to the SDS-polyacrylamide gel. The high and low molecular weight forms of apo B, identified by comparing their mobilities with those of apo B from LDL and chylomicrons, are designated as B-100 and B-48, respectively (35).

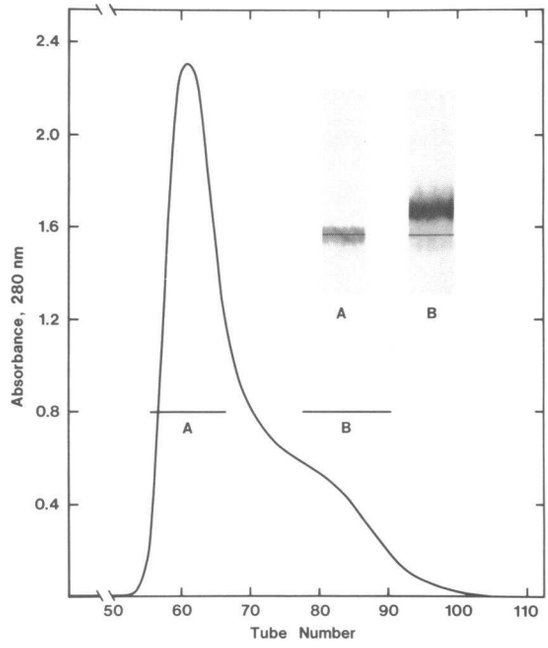

Figure 2. Gel filtration permeation chromatography of R.M.'s $d < 1.006$ lipoproteins. The $d < 1.006$ fraction (21 mg of lipoprotein protein) was subfractionated on an agarose (A-5m) column (2.5 × 100 cm). Fractions *A* and *B* were taken as indicated. Inset: paper electrophoretograms of fractions *A* and *B*.

isoelectric focusing. As shown in Fig. 5, the isolated E3 band moved one charge position after cysteamine modification, while the E1 moved two charge positions, indicating that the E3 and E1 contained one and two residues of cysteine per mole, respectively. The cysteine content of the E3 and E1 was confirmed by amino acid analysis (Table III). Overall, the compositions of both the E3 and E1 isoforms were in general agreement with that of apo E3 (5, 6) and, although the results are not conclusive, they suggest possible differences between E3 and E1 of several amino acids (e.g., arginine, cysteine, and aspartic acid), which will be shown to be important.

To pursue the identification of the amino acid substitution(s), the apo E3/1 was subjected to cyanogen bromide digestion, and the fragments were isolated as previously described (6). The 17-residue CB4 peptide and the 93-residue CB5 peptide were subjected to sequence analysis. The sequence of CB4 was identical to that of apo E2 and apo E3 CB4 (6), and established that both the E3 and E1 from subject R.M. contained cysteine at residue 112 (data not shown).

Peptide CB5 (29% overall yield) from the apo E3/1 of subject R.M. had the following composition (in residues per mole): Asp 4.4, Thr 2.2, Ser 3.9, Glu 16.3, Pro 2.1, Gly 8.3, Ala 13.1, Cys (as cysteic acid) 0.6, Val 5.4, Met (as homoserine lactone) 0.6, Ile 0.9, Leu 14.2, Tyr 0.8, Lys 3.3, His 1.0, and Arg 14.1. The sequence of peptide CB5 was revealing, in that at cycle 33 (residue 158) both cysteine and arginine were observed (Table IV), indicating that the second cysteine residue in the E1 was located at residue 158, and that the E3 contained arginine at this position. The remaining sequence of the apo E3/1 CB5, up to residue 172, was identical to that reported for apo E2 or E3 (6).

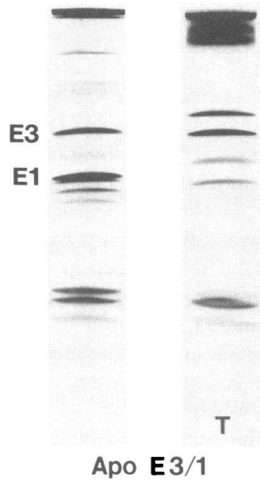

Figure 3. Isoelectric focusing on polyacrylamide gels of control and cysteamine-modified VLDL from subject R.M. Approximately 150 μg of VLDL protein were applied to each gel. The control sample was reduced with 0.1% β-mercaptoethanol before its application to the gel. The cysteamine modification of the VLDL (T) was carried out on the VLDL before delipidation as previously described (5). The pH range of the ampholytes used was 4–6.

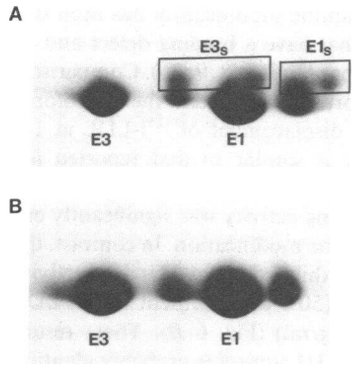

Figure 4. Two-dimensional polyacrylamide gel electrophoresis of apo E3/1 from subject R.M. (*A*) Control apo E3/1; (*B*) neuraminidase-digested apo E3/1. Isoelectric focusing over the pH range of 4–6 was carried out in the first dimension. The gel was then incubated in 0.02 M Tris containing 1% SDS, 10% glycerol, and 1% β-mercaptoethanol (pH 8.2) and applied to the top of the second dimension 12.5%-SDS slab gel. The brackets enclose bands designated $E3_s$ and $E1_s$, which represent sialylated forms of E3 and E1. The two bands with the same apparent molecular weight as E3 and E1 that migrate one charge position more acidic relative to these isoforms are commonly observed in our laboratory with purified apo E preparations. These bands were absent in the two-dimensional gels of freshly isolated VLDL (data not shown). Presumably, they were generated during subsequent isolation procedures. This is analogous to the results observed with apo A-I; it has been suggested that the additional acidic isoforms result from partial deamidation during storage and isolation (41, 42).

*Table III. Amino Acid Composition of the E1 and E3 Isoforms Isolated from Apo E3/1**

	E1‡	E3§	Residue difference	Apo E3/3‖
Asp	13.5	12.8	0.7	12
Thr	10.7	10.7	0	11
Ser	12.9	12.8	0.1	14
Glu	70.4	70.2	0.2	71
Pro	9.2	9.6	0.4	8
Gly	16.8	17.1	0.3	17
Ala	33.7	33.9	0.2	35
Val	22.3	23.0	0.7	22
Met	6.2	6.3	0.1	7
Ile	2.2	2.4	0.2	2
Leu	37.3	36.7	0.6	37
Tyr	4.3	4.3	0	4
Phe	3.7	3.6	0.1	3
Trp	ND	ND	—	7
Lys	12.3	11.9	0.4	12
His	2.2	2.1	0.1	2
Arg	32.4	33.5	1.1	34
Cys¶	1.9	1.1	0.8	1

ND, not determined.
* Expressed as number of residues per mole.
‡ Average of eight determinations.
§ Average of three determinations.
‖ From sequence, reference 6.
¶ Determined after performic acid oxidation, duplicate determinations.

Normally the CB5 fraction is slightly contaminated with a small quantity of the partial digestion fragment CB4,5 (6). This results in small amounts of aspartic acid (contributed by CB4, residue 110) appearing along with Gly_{127} at cycle 2 in the degradation of CB5. With the CB5 of this apo E3/1, however, the yield of aspartic acid was too high to be accounted for by CB4,5 contamination. These data suggested that aspartic acid was substituted for glycine in the E1 at residue 127. To confirm this, the E1 isoform (7.5 mg) was subjected to tryptic digestion, and the tryptic peptide designated T5c containing this region of the protein was isolated (corresponding to peptide T15, residues 120–134, as shown in reference 6). The electrophoretic mobility of T5c from this apo E1 at pH 6.4 (m6.4 = −0.44 for T5c compared with m6.4 = −0.30 for T15) indicated that it had an additional negative charge relative to T15 from apo E2 (Table V and reference 6). Amino acid analysis clearly demonstrated a glycine-aspartic acid difference relative to the apo E2 peptide (Table V and reference 6). Sequence analysis revealed aspartic acid at cycle 8, demonstrating that aspartic acid was substituted for glycine at residue 127. The remainder of the T5c sequence was identical to T15 from apo E2 (Table V and reference 6). In addition, the amino acid compositions and/or sequences of all the remaining tryptic fragments from E1, except T2 and T38 (nomenclature from reference 6), were examined and found to be identical to those reported for apo E2 (data not shown), indicating that other amino acid substitutions were unlikely.

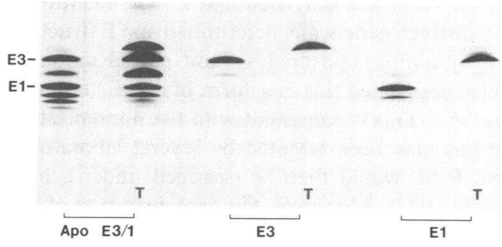

Figure 5. Isoelectric focusing on polyacrylamide gels of column-isolated apo E3/1 and the isolated E3 and E1 isoforms from subject R.M. The control samples were reduced with 0.1% β-mercaptoethanol and the treated samples (T) were modified with cysteamine. The E3 and E1 isoforms were isolated by preparative isoelectric focusing of the column-isolated apo E3/1 as described in Methods. The gels were focused over the pH range 4–6.

Additional characterization of the apo E1 included an examination of its ability to bind to apo B,E (LDL) receptors on cultured human fibroblasts. When combined with DMPC, the apo E3/1 was only moderately effective compared with apo E3 in displacing ^{125}I-LDL from fibroblast receptors (~30% of the apo E3 binding activity) (Fig. 6 *A*). Cysteamine modification resulted in a 2.2-fold increase in receptor activity. This increase

Table IV. Partial Sequence Analysis of Peptide CB5 from Subject R.M. Apo E3/1

Residue number	Cycle number	Amino acid identified	nmol	$(n + 1)/n$
	0		125.0	
126	1	Leu	44.0	0.11
127	2	Gly (Asp)	32.6 (16.6)	0.11 (0.16)
128	3	Gln	12.8	0.26
129	4	Ser	28.5	0.17
130	5	Thr	35.0	0.15
131	6	Glu	26.7	1.31
132	7	Glu	34.9	0.24
133	8	Leu	45.8	0.26
134	9*	Arg	6.6	ND
135	10	Val	45.4	0.27
136	11*	Arg	7.7	ND
137	12	Leu	40.8	0.34
138	13	Ala	44.3	0.26
139	14	Ser	20.5	0.29
140	15*	His	4.2	ND
141	16	Leu	40.8	0.36
142	17	Arg	4.3	ND
143	18	Lys	14.5	0.23
144	19	Leu	37.8	0.38
145	20*	Arg	3.9	ND
146	21	Lys	14.5	0.22
147	22	Arg	4.4	ND
148	23	Leu	35.3	1.12
149	24	Leu	39.4	0.41
150	25*	Arg	4.7	ND
151	26	Asp	5.8	0.46
152	27	Ala	30.4	0.55
153	28	Asp	5.9	1.32
154	29	Asp	7.8	0.41
155	30	Leu	28.4	0.53
156	31	Gln	6.2	0.58
157	32	Lys	8.4	0.46
158	33*	Arg* (Cys)	1.8* (2.7)	ND (0.55)
159	34	Leu	23.1	0.55
160	35	Ala	20.6	0.58
161	36	Val	16.9	0.53
162	37	Tyr	9.2	0.52
163	38	Gln	3.7	0.67
164	39	Ala	18.4	0.66
165	40	Gly	14.7	0.46
166	41	Ala	16.6	0.69
167	42*	Arg	2.0	ND
168	43	Glu	7.2	0.81
169	44	Gly	8.8	0.82
170	45	Ala	12.1	0.89
171	46	Glu	6.4	0.87
172	47*	Arg	1.5	ND

ND, not determined.
* From aqueous phase analysis.

in binding activity after cysteamine modification has been observed in all apo E variants that have a binding defect and in which cysteine is substituted for arginine (8, 9, 36). Comparison of the isolated E1 and E3 isoforms revealed that the E1 isoform was markedly defective (50% displacement of ^{125}I-LDL at 1.1 μg/ml). This binding activity is similar to that reported for apo E2(Arg_{158} → Cys) (36). Furthermore, as is the case with E2(Arg_{158} → Cys), the E1 binding activity was significantly enhanced (5.1-fold) after cysteamine modification. In contrast, the E3 isoform from subject R.M. exhibited normal binding activity compared with an E3 control (50% displacement of ^{125}I-LDL at a concentration of 0.044 μg/ml) (Fig. 6 *B*). These results indicate that the E3 from this E3/1 subject is probably identical in structure to apo E3.

Discussion

The results from several apo E phenotyping studies (3, 10–14) have indicated that all subjects can be classified under one of six phenotypes (E2/2, E3/3, E4/4, E4/3, E4/2, and E3/2), as predicted from the three allele model of Zannis and Breslow (3, 4). Although structural heterogeneity has been shown to exist within the E2/2 phenotype (8, 9) and phenotypes other than the six commonly described could predictably exist, none of the screening studies to date have described a new phenotype. In the present study, however, we describe and characterize such a phenotype (apo E3/1). An apo E variant that focuses with an isoelectric focusing point similar to the apo E1 described in this study has also been observed by Gregg et al. (25), and they have identified two phenotypes that appear to be equivalent to E3/1 and E2/1. Recently, in our apo E screening studies, an unrelated subject with the E4/1 phenotype was identified (data not presented).

The subject, R.M., is heterozygous for E3 and for a band that focuses in the E1 position on isoelectric focusing gels. Normally, only sialylated derivatives of the major isoforms focus in this position. Two-dimensional gel analysis, performed in conjunction with neuraminidase treatment, ruled out the possibility that the E1 band was sialylated apo E. The identity of the E1 band as a distinct, genetically determined apo E structure was established by amino acid analysis and partial sequence analysis. We have designated this new form of apo E "E1" and its gene (allele) "ε1." This is consistent with the nomenclature for apo E that has now been adopted by several laboratories (7). The subject R.M. would then be classified under a new, seventh phenotype, E3/1. Moreover, the demonstration of this new, charged form raises the possibility that other charged variants exist (either with isoelectric focusing points more acidic than E1 or more basic than E4), and that other phenotypes will be described. Studies are now underway to determine the frequency with which the ε1 allele occurs in the Finnish population, the nationality of subject R.M. Vertical transmission of the ε1 allele has been observed in one of the proband's sons, who also has the E3/1 phenotype.

Table V. Sequence and Amino Acid Analysis of Tryptic Peptide T5c from E1 Isoform (Subject R.M.)

Peptide T5c (yield 36%; ml.9 = 0.55, m6.4 = −0.44)

Analysis:	1.0	5.0	1.0		1.1	0.9	2.0	1.2		0.9	0.9				1.0
	(Gly) -	Glu -	Val -	Gln -	Ala -	Met -	Leu -	Asp -	Gln -	Ser -	Thr -	Glu -	Glu -	Leu -	Arg
Sequence:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(*nmol*) (18)	ND	7.0	7.0	2.8	5.5	3.4	4.6	3.0	1.3	1.0	0.4	2.0	3.1	1.2	1.6*
$(n + 1)/n$	ND	0.12	0.09	0.17	0.16	0.24	0.28	0.24	0.69	0.32	0.60	—	0.64	ND	ND

ND, not determined. * Determined by amino acid analysis (no hydrolysis) after recovery of material from sequencer following cycle 14.

Partial sequence analysis of the new apo E variant revealed that it differs from E2(Arg_{158} → Cys) at residue 127, where aspartic acid is substituted for glycine. From analysis of all but two of the tryptic peptides, there was no indication that other differences exist between this apo E1 and E2(Arg_{158} → Cys). Although it cannot be completely ruled out that there are other amino acid substitutions in E1, the aspartic acid/glycine interchange at residue 127 is sufficient to account for the difference of one negative charge observed between E1 and E2(Arg_{158} → Cys) on isoelectric focusing gels. Based on the nomenclature (9), which designates apo E variants by both their isoelectric focusing position and structure relative to the parent form (apo E3), this variant has been designated E1(Gly_{127} → Asp, Arg_{158} → Cys). The amino acid interchange at residue 127 represents a new substitution site in apo E. Because the E1 variant also contains a substitution previously identified in apo E2(Arg_{158} → Cys), which is specified by the $\epsilon 2$ allele, it is probable that the $\epsilon 1$ allele arose as a result of a point mutation in the $\epsilon 2$ allele.

Amino acid substitutions in apo E at residues 145, 146, and 158 (8, 9, 36) have been shown to diminish apo E receptor binding activity. In contrast, the cysteine/arginine interchange at residue 112, which differentiates E3 and E4(Cys_{112} → Arg), has no effect on the ability of apo E4 to bind to apo B,E receptors. Determination of the binding activity of E1(Gly_{127} → Arg, Arg_{158} → Cys) has revealed that the magnitude of the defect is similar to that of E2(Arg_{158} → Cys). This would suggest that the glycine/aspartic acid interchange at residue 127 has little or no effect on receptor binding and that the defective receptor activity is due entirely to the cysteine substitution at residue 158. This is not an unreasonable assumption, since residue 127 lies outside of the region of apo E that is critical for receptor interaction (37, 38). Furthermore, up to now all substitutions that have been shown to affect receptor activity involve the replacement of arginine or lysine residues by neutral amino acids. This latter point is consistent with previous studies in which selective chemical modification of lysine and arginine residues was shown to influence the ability of apo E to bind to fibroblast receptors. It has been demonstrated that selective modification of arginine residues with 1,2-cyclohexanedione (39), and lysine residues by carbamylation, acetoacetylation, or reductive methylation (40), abolishes the receptor binding activity of apo E.

Because of the limited availability of family members, it has not been possible to determine if the E1 variant and the hypertriglyceridemia cosegregate. This leaves open the question of whether the defective receptor binding activity of the E1

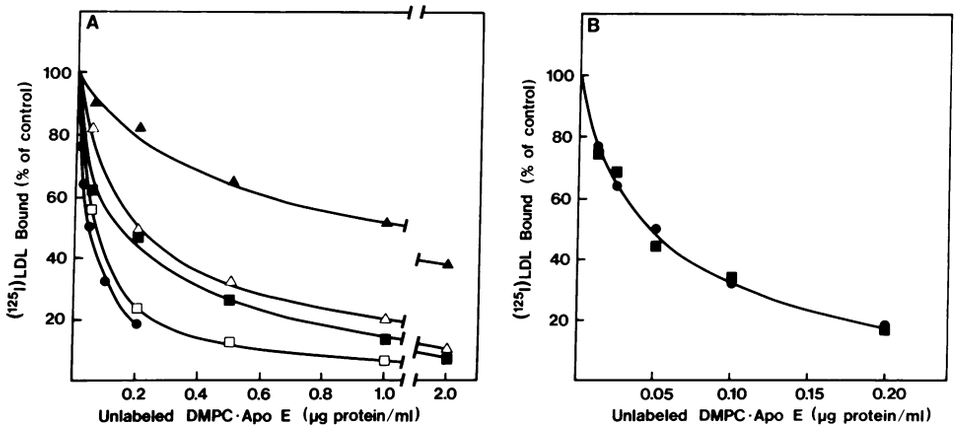

Figure 6. Ability of various apo E·DMPC recombinants to compete with ^{125}I-LDL for binding to apo B,E receptors on cultured fibroblasts. The isolated DMPC recombinants were mixed at various protein concentrations with ^{125}I-LDL (2 μg), and the mixtures were incubated with cultured fibroblasts on 35-mm culture plates for 4 h at 4°C. The 100% value for the ^{125}I-LDL was 100 ng bound/mg of cellular protein. (*A*) Apo E3/1 of subject R.M. (■); cysteamine-modified apo E3/1 of subject R.M. (□); E1 isoform of subject R.M. (▲); cysteamine-modified E1 isoform of subject R.M. (△); and apo E3/3 control (●). (*B*) E3 isoform of subject R.M. (■) and apo E3/3 control (●).

variant is the cause, or a contributing factor, to the hyperlipidemia. The complexity of the lipoprotein abnormalities in subject R.M. prevents a precise characterization of the hyperlipoproteinemia. However, it appears that the subject does not clear chylomicron remnants normally (as suggested by the β-VLDL and apo B-48 in the $d < 1.006$ fraction) and has problems converting VLDL into LDL (despite normal levels of lipase and no obvious deficiency in apo C-II). Although the subject is heterozygous (E3/1), the possibility that the abnormal E1 variant might be involved in the hyperlipoproteinemia cannot be ruled out. It has now been shown that certain subjects heterozygous for apo E2 display type III hyperlipoproteinemia (9, 22). More detailed studies of R.M.'s lipoproteins and his family will be required to resolve this question.

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