Characterization of a Unique Human Apolipoprotein E Variant Associated with Type III Hyperlipoproteinemia*

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Vassilis I. Zannis and Jan L. Breslow
From the Division of Metabolism, Children's Hospital Medical Center, Boston, Massachusetts 02115 and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

Human very low density lipoprotein (VLDL), purified by ultracentrifugation from the plasma of 47 subjects, was analyzed by a two-dimensional gel electrophoresis system designed to achieve maximum separation of the apolipoprotein E (apo-E) isoproteins. This analysis revealed many apo-E isoproteins differing from each other in both size and charge, as well as genetic variations in the overall electrophoretic pattern of apo-E isoproteins. The patterns of VLDL apo-E isoproteins from different individuals were of two major types referred to as classes α and β. In the α pattern, at least seven different groups of isoproteins were seen; each contained one or more proteins with the same isoelectric point that differed slightly in molecular weight. The β pattern was similar to the α pattern but had a greatly diminished concentration of one isoprotein which was a major component of the α pattern. Of the 47 subjects examined, 36 were α and 17 were β. When VLDL apo-E obtained from different subjects with the α pattern was compared in mixing experiments, the electrophoretic patterns did not always coincide. Using this mixing technique, at least two subclasses of class α were identified. These have been designated αII and αIII and differ from each other by 1 charge unit. Similar mixing experiments with VLDL apo-E from individuals with the β pattern revealed two common subclasses designated βII and βIII differing from each other by 1 charge unit. Three subjects with type III hyperlipoproteinemia had a unique acidic β pattern of apo-E designated subclass βIVa. This βIVa subclass of apo-E showed no deficiency or altered ratio of any of the isoproteins that comprise the βII or βIII subclasses of apo-E, but did exhibit a pattern that differed from the common subclasses βII and βIII by approximately 2.5 and 1.5 negative charges, respectively. The discovery of subclasses of apo-E could allow a better understanding of human diseases which may be associated with apo-E abnormalities. This report indicates that a rare apo-E variant (apo-E subclass βIVa) may be the underlying molecular defect in type III hyperlipoproteinemia, a genetic disease which causes premature atherosclerosis.

In 1974, apolipoprotein E was identified in normal human VLDL1 (1). This protein has a molecular weight of approximately 33,000 and an amino acid composition enriched in arginine (2). In humans, a diet containing cholesterol increases plasma apo-E levels some of which is found in the HDL density range of 1.063 to 1.21 g/ml (3, 4). The HDL fraction containing apo-E can be quantitatively precipitated from plasma with hepatic and manganese oxide has been shown to have a greater affinity than LDL itself for the LDL receptors of human skin fibroblasts (5-8). Plasma apo-E levels are increased in human type III hyperlipoproteinemia (9-11). Multiple apo-E isoproteins have been reported (12-14). Utermann et al. found three isoproteins of apo-E (apo-E-I, apo-E-II, and apo-E-III), and he reports that individuals with type III hyperlipoproteinemia are deficient in the apo-E isoprotein, apo-E-III (15-15). These studies, as well as others, suggest that apo-E plays an intimate role in regulating plasma cholesterol metabolism, and perhaps in atherogenesis itself.

To understand better apo-E metabolism, and any possible association of this apoprotein with human disease, we studied human plasma apo-E using high resolution, two-dimensional gel electrophoresis. In this report, we show that human apo-E consists of several isoproteins differing in size or charge, or both. We also demonstrate that genetic variability may exist in apo-E and that a rare apo-E variant is associated with type III hyperlipoproteinemia.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, ovalbumin, lysozyme, trypsin inhibitor, Trypsin, glycine, sodium bisulfate, and agarose were purchased from Sigma Chemical Co. Ampholines (pH 2.5 to 4 and 5 to 8) were obtained from LKB. Nonidet P-40 was purchased from Particle Data Laboratories, Ltd. Sodium dodecyl sulfate manufactured by British Drug House Chemical Ltd was purchased through Golland-Schleiniger. Acrylamide, bisacrylamide, N,N',N,N'-tetramethylenediamine, ammonium persulfate, Comassie brilliant blue, bromphenol blue, and Biolites (ampholines, pH 4 to 6) were obtained from Bio-Rad. Urea ultrapure grade was a product of Schwarz/Mann. All other materials were of the purest grade commercially available.

Plasma VLDL Separation and Apo-E Purification—Blood was drawn from volunteers after they had fasted for 10 to 12 h. VLDL was isolated from 5 ml of plasma after overlaying the specimen with approximately 1 ml of normal saline and centrifugation in a Beckman LS-65 ultracentrifuge for 18 h at 120,000 × g in a 40.2 rotor. This VLDL was used directly as a source of apo-E for two-dimensional analysis without delipidation, although delipidation of VLDL prior to analysis with chloroform/methanol (2:1) did not affect the observed apo-E isoprotein pattern. In a few experiments, the VLDL obtained after a single ultracentrifugation was further purified by recentrifugation under the conditions specified above. This purified VLDL was used for complete apo-E purification by published procedures (2, 15)."
were 17 × 20.5 cm with a thickness of 0.75 mm. The separating gel (12%: 11.7% acrylamide, 0.32% bisacrylamide) and the stacking gel (4.5%: 4.4% acrylamide, 0.12% bisacrylamide) were prepared according to the method of Davis (20). Protein molecular weight markers were introduced in the second dimension as follows: bovine serum albumin, 68,000; ovalbumin, 43,000; bovine purine nucleoside phosphorylase, 30,000; human apo-A-I, 28,000; trypsin inhibitor, 19,000; and egg white lysozyme, 14,300. Iodination and one-dimensional chymotryptic peptide mapping of apo-E isoprotein spots cut from the dried two-dimensional gel was performed by published techniques (21-23).

The estimation of charge unit differences between apo-E isoproteins was done by boiling apo-E in 9 M urea and 20% ampholytes (pH 3.5 to 10) for 5 min prior to two-dimensional electrophoresis analysis. This resulted in carbamylation of free amino groups generating new isoproteins differing from the original isoprotein by 1 or more negative charge units (24). The distances of these carbamylated isoproteins from the isoprotein of origin indicates how far apart two isoproteins differing by 1, 2, etc. charge units will focus.

RESULTS

Definition of Apo-E Isoprotein Classes and Presentation of a New Descriptive Nomenclature of Apo-E Isoproteins—VLDL apo-E exists in two major two-dimensional electrophoresis patterns shown in Fig. 1. A and B with a schematic drawing of these patterns displayed in Fig. 1C. The complexity of these apo-E patterns requires that new nomenclature be introduced for descriptive purposes. The apo-E patterns shown in Fig. 1, A and B have been named apo-E classes α and β, respectively. Each apo-E class contains numerous protein components called isoproteins of apo-E that differ from each other in isoelectric point or molecular weight, or both. These apo-E isoproteins are distributed in at least seven isoprotein groups numbered 1 through 7 (Fig. 1C). The individual isoproteins in an isoprotein group have also been as-

Fig. 1. Two-dimensional gel electrophoresis of VLDL apo-E patterns. Only the area of the gel in the vicinity of apo-E is shown. Panel A, 25 μg of apo-E class α; Panel B, 40 μg of apo-E class β; Panel C, schematic comparison of α and β patterns; and Panel D, autoradiogram of one-dimensional chymotryptic peptide maps of human apo-E isoproteins of class α and of purified apo-E and apo-A-I. Iodination and mapping of apo-E isoprotein spots cut from the dried gel was performed as described in Refs. 21 to 23. Lanes a to k contain the following samples: a, isoprotein 1; b, isoprotein 2; c, isoprotein 3; d, isoprotein 4; e, isoprotein 4'; f, isoprotein 5; g, isoprotein 5'; h, the isoproteins in group 6; i, the isoproteins in group 7; j, purified apo-E class α; k, human apo-A-I. Note the peptide similarities of apo-E isoproteins and their differences from the peptides of apo-A-I. The apo-E isoprotein peptides of the other apo-E subclasses including those of type III patients are essentially identical with those shown in this figure. In this and all subsequent photographs, the cathode is on the left and the anode is on the right of the photograph.
Unique Apo-E Variant in Type III Hyperlipoproteinemia

signed numbers. The group number indicates the single isoprotein component of the group (for groups 1 to 3) or the isoprotein with the lowest molecular weight (for groups 4 through 7). When an isoprotein group contains more than one isoprotein, those of higher molecular weight are characterized by primed numbers (e.g. 4', 5', 5''); see Fig. 1C). The isoproteins shown in Fig. 1. A and B were all apo-E by the following criteria. 1) Our work and published work of others show that apo-E is a major constituent of VLDL and on one-dimensional SDS-gel electrophoresis is a broad band. The molecular weight of this broad band is reported to be either 33,000 or 39,900 in different laboratories (2, 5, 13, 14, 16). No other major or minor VLDL apoproteins besides apo-E have been reported in this molecular weight region; and 2) apo-E, purified by published methods (2, 16), and each of the isoproteins of both class a and class β (Fig. 1, A and B) have identical one-dimensional chymotryptic peptide maps (Fig. 1D). A comparison of the apo-E isoproteins in classes α and β shows that the major difference is that apo-E class β has a greatly diminished apoprotein component 4.

Subclasses of Apo-E Isoproteins—Two-dimensional gel electrophoresis analysis of mixtures of VLDL obtained from different subjects with α patterns of apo-E isoproteins revealed that α patterns were not always superimposable (Fig. 2A). A similar observation was made in mixing experiments with VLDL from subjects with β patterns of apo-E isoproteins (Fig. 2B). A systematic comparison of VLDL apo-E from all normal subjects studied revealed two subclasses of class α (αII and αIII) and two subclasses of class β (βIII and βIVa). These mixing experiments established that in different individuals the entire pattern of apo-E isoproteins could be shifted in charge but maintain the same quantity of isoproteins in the same relative concentrations. Of the 44 normal subjects studied, 13 were αII, 17 were αIII, 2 were βII, and 12 were βIII. The apo-E isoprotein subclass of a given individual remained the same even after delipidation of VLDL, purification of apo-E, or repeat plasma sampling on different days of the same subject.

A Unique Subclass of Apo-E in Type III Hyperlipoproteinemia—A two-dimensional gel electrophoresis analysis of VLDL apo-E from three patients with type III hyperlipoproteinemia was also performed. The three patients had a broad β band on lipoprotein electrophoresis, ratios of VLDL cholesterol/triglyceride of 0.41, 0.44, and 0.47, and clinical histories of premature vascular disease, all of which confirmed the diagnosis of type III hyperlipoproteinemia (25-28). These patients had a β pattern of VLDL apo-E without any missing or altered apo-E isoproteins (Fig. 3A). However, mixing experiments indicated that the whole pattern in these patients was shifted by approximately 2.5 and 1.5 negative charges, relative to subclasses βII and βIII, respectively (Fig. 3B and C). This unique subclass of apo-E has been designated as βIVa. The one-dimensional chymotryptic peptide maps of all the isoproteins of apo-E subclass βIVa are identical with the maps of the isoproteins in the common apo-E subclasses (data not shown). The relative charge relationship of all the apo-E subclasses is shown schematically in Fig. 4.

**DISCUSSION**

Recently, apo-E has been studied intensively because of its association with lipoproteins affecting cholesterol metabolism.

**Fig. 2.** Two-dimensional gel electrophoresis patterns of mixtures of VLDL fractions obtained from individuals with different apo-E subclasses are shown. Panel A, mixture of 30 μg of αII and 20 μg of αIII apo-E; Panel B, mixture of 25 μg of βII and 25 μg of βIII apo-E. The contribution of each subclass to the overall pattern is indicated. However, several components result from merging of isoproteins belonging to both apo-E subclasses.

**Fig. 3.** Two-dimensional gel electrophoresis of VLDL obtained from a patient with type III hyperlipidemia and a mixture of his VLDL and normal VLDL fractions belonging to two different apo-E subclasses are also shown. Panel A, 40 μg of type III apo-E; Panel B, 35 μg of αII and 35 μg of βIVa apo-E; and Panel C, 30 μg of βII, 15 μg of αIII, and 15 μg of βIVa apo-E. The contribution of each subclass to the overall pattern is indicated. However, some components result from merging of isoproteins belonging in different apo-E subclasses.

**Table 1.** APO E SUBCLASSES CHARGE SHIFT

<table>
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<th>SUBCLASSES</th>
<th>CHARGE SHIFT</th>
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</thead>
<tbody>
<tr>
<td>α II</td>
<td>0</td>
</tr>
<tr>
<td>α III</td>
<td>-1</td>
</tr>
<tr>
<td>β II</td>
<td>0</td>
</tr>
<tr>
<td>β III</td>
<td>-1</td>
</tr>
<tr>
<td>βIVa</td>
<td>-2.5</td>
</tr>
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**Fig. 4.** Schematic presentation of the charge relationship of human apo-E subclasses.
isoproteins are products of different genes but rather that they are caused by post-translational modifications. Post-in the human population. This question is being explored through studies of apo-E patterns in members of large kindred. 

This report documents much greater complexity of apo-E than has been previously seen. We describe numerous apo-E isoproteins with differences in isoelectric point or molecular weight, or both. A few apo-E isoproteins with different isoelectric points have been reported (12-15) but the size heterogeneity in apo-E isoproteins has been overlooked previously in spite of the commonly seen broad apo-E band on one-dimensional SDS-polyacrylamide gel analysis (2, 5, 13, 14, 16). All of the apo-E isoproteins described here have the same one-dimensional chymotryptic peptide maps which suggest that they have a similar but not necessarily identical primary amino acid sequence. It is unlikely that all of these isoproteins are products of different genes but rather that they are caused by post-translational modifications. Post-translational modification of proteins is a widespread phenomenon in nature (18, 29-32) and such modifications of apo-E may play an important but as yet undefined role in plasma lipoprotein physiology.

The discovery of apo-E classes and subclasses indicates that genetic polymorphism of the gene specifying apo-E may exist in the human population. This question is being explored through studies of apo-E patterns in members of large kindred.

Three patients with type III hyperlipoproteinemia were shown to have a unique VLDL apo-E isoprotein subclass designated β1Va. This apo-E subclass showed no deficiency or altered ratio of any of the isoproteins that comprise the apo-E subclass βII or βIII. Instead, the entire pattern is shifted 2.5 and 1.5 negative charges relative to apo-E subclasses βII and βIII, respectively. In recent work, Utermann et al. have separated isoproteins of apo-E by one-dimensional isoelectric focusing in 8 M urea. They have specified these isoproteins apo-E-I, apo-E-II, apo-E-III, and apo-E-IV (present only in 27% of subjects), in order from acidic to basic, and have found a large number of individuals with type III hyperlipoproteinemia deficient in apo-E-III (13-15). They also found normal individuals in the population deficient in apo-E-III and concluded that apo-E-III deficiency was a necessary but not sufficient condition for type III hyperlipoproteinemia (15). As can be seen in Fig. 4 (and Utermann's original one-dimensional isoelectric focusing data), the apo-E pattern of type III hyperlipoproteinemia patients, subclass βIIa, is shifted to more acidic isoelectric points relative to all normal apo-E subclasses. This pattern could, therefore, be interpreted as deficient in a basic apo-E isoprotein. In addition, a basic apo-E isoprotein would appear to be missing if a sample of apo-E subclass βII were analyzed in a one-dimensional system along with many samples of apo-E subclass βII. Similarly, comparisons of apo-E subclasses αII or βIII to apo-E subclass αI would suggest a missing apo-E isoprotein. Thus, we can explain the necessary but not sufficient nature of the association of an apparent apo-E isoprotein deficiency (as proposed by Utermann et al) with type III hyperlipoproteinemia. It appears that what has previously been called homonymous apo-E-III deficiency (13-15) is on our system a charge shift, not an isoprotein deficiency. This charge shift most likely results from a structural mutation in the apo-E gene. This rare genetic variant of apo-E may underlie type III hyperlipoproteinemia.

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V I Zannis and J L Breslow


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