Identification and Characterization of a New Variant of Apolipoprotein E (apo E-Kochi)

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A new variant of apolipoprotein E (apo E), named apo E-Kochi, was identified in the sera of a 29-year-old male with hyperlipoproteinemia as characterized by a broad-beta band. The characteristic double bands of apo E were seen in the isoelectric focusing gel of very low density lipoprotein from the proband and three members of his family. Of the double bands from the probands, the more cationic component was identical to ordinary apo E3 and the other anionic band was located at approximately a distance of one-half charge to the anode side. This anionic band is a new electrophoretic isoform of apo E (apo E-Kochi), and the molecular weight by sodium dodecyl sulfate electrophoresis and its antigenicity against anti apo E serum are the same as apo E3. Sequence analysis of lysyl endopeptidase fragments showed that apo E-Kochi differs from normal apo E3 at residue 145, where an arginine residue is substituted for histidine.

Key words: Hyperlipoproteinemia, Broad-beta band

Apolipoprotein E (apo E) is a polypeptide composed of 299 amino acid residues. It shows genetically-determined polymorphism (1, 2), and it has been demonstrated that the major isoforms (apo E4, E3 and E2) can be detected in an isoelectric focusing gel (3). The most common of these isoforms is apo E3. In addition, sialylation of these isoforms creates minor isoforms (4). Apo E4, E3 and E2 are synthesized from three types of alleles depicted as $\epsilon 4$, $\epsilon 3$ and $\epsilon 2$ (3). As a result of the various combinations of these alleles, six different phenotypes have been observed to exist (3). Moreover, it is known that some persons with the apo E2/2 phenotype develop type III hyperlipoproteinemia (5).

In recent years, a number of apo E variants have been discovered (6–17). In the present study, we describe the identification and characterization of a new variant of apo E, designated as apo E-Kochi, which was discovered in a hyperlipoproteinemic patient with a broad-beta band; included are the findings of serum lipid and lipoprotein analyses, and

the results of studies of family members.

MATERIALS AND METHODS

Analysis of serum lipids, lipoproteins and apolipoproteins

Serum or plasma (EDTA) was obtained from the proband and his relatives after an overnight fast. The lipoprotein fractions were isolated from the plasma (sera except the proband) by sequential ultracentrifugations using NaBr and NaCl for density adjustment (18). The concentrations of cholesterol, triglycerides and phospholipids in the serum and each lipoprotein were measured enzymatically with kits (Kyowa Medex Co., Ltd., Tokyo, Japan). Agarose electrophoresis of the serum was carried out in accordance with the method of Pagnan et al (19). The concentrations of apolipoproteins A-I, A-II, B, C-II, C-III and E were measured with single radial immunodiffusion assay kits (Daiichi Kagaku Co., Ltd., Tokyo, Japan) (20).

Post-heparin lipolytic activity

The lipolytic activity in post-heparin plasma was

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determined by selective measurement of the extrahepatic lipoprotein lipase (LPL) and hepatic triglyceride lipase (H-TGL) activities in accordance with the method of Nozaki et al (21).

Analysis of apo E and isoform

Very low density lipoprotein (VLDL) was delipidated with ethanol: acetone (1:1), and washed with ether and then dissolved in a Tris-HCl buffer (pH 8.6) containing 8 mol/l urea and 0.01 mol/l dithiothreitol. Analytical isoelectric focusing of apo VLDL was carried out using a 7.5% polyacrylamide disc gel (0.5 cm × 10 cm) containing 8 mol/l urea and 2% Ampholine with a pH range of 4–6 (LKB-Produkter AB; Bromma, Sweden) (22). The digestion of apo E by neuraminidase (Type IV; Sigma Chemical Co., St. Louis, MO, USA) was carried out at 37°C for 3 h in a 0.1 mol/l acetate buffer (pH 5.0). The ratio of the enzyme to the VLDL protein was 1:30. The modification of apo E with cysteamine was conducted according to the method of Weisgraber et al (23).

Two-dimensional electrophoresis was performed by the method of O'Farrell (24); the concentrations of Ampholine for the isoelectric focusing of the first dimension were adjusted to 0.4, 1.2 and 0.4% for the 4–6, 5–7 and 6–8 pH ranges, respectively, while a sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel as employed for the second dimension.

The purification of each apo E isoform was carried out as follows. The disc gel after isoelectric focusing was separated in half along its vertical axis. One half was then fixed and stained with Coomassie Brilliant Blue G 250, while sites corresponding to each of the isoform bands were cut out of the other half of the gel. From pieces of the gel, each of the apo E isoforms was electrophoretically eluted into a dialysis sack (25) and dialyzed against 0.1 mol/l NH_4HCO_3 . These apo E isoforms were then reacted with anti-human apo E goat serum (Daiichi Kagaku Co., Ltd., Tokyo, Japan) by the double immunodiffusion method (Ouchterlony method).

Sequence analysis

The $d < 1.020$ lipoprotein fraction from the proband was delipidated with chloroform and methanol (2:1) and the apoprotein mixture was solubilized in 6 mol/l guanidine containing 0.1 mol/l Tris-HCl and 0.01% EDTA (pH 7.4). Apo E was

purified from the apoprotein mixture by gel filtration on Sephacryl S-300 HR (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in a column (2.4 × 100 cm) equilibrated with 4 mol/l guanidine containing 0.1 mol/l Tris-HCl, 0.1% β -mercaptoethanol, and 0.1% EDTA (pH 7.4). Pooled fractions were dialyzed against 5 mmol/l NH_4HCO_3 and lyophilized.

Lysyl endpeptidase digestion of purified apo E (300 μg) from the proband was carried out using a substance to enzyme ratio of 50:1 in 0.01 mol/l Tris-HCl (pH 9.0) for 4 h at 37°C after incubation in 8 mol/l urea solution for one h at room temperature.

The peptides produced by digestion were separated by reverse phase high performance liquid chromatography (HPLC) using a Vydac C18 column (Senshu Pak VP318) and a trifluoroacetic acid (TFA) buffer system (buffer A, 0.1% TFA in H_2O ; buffer B, 0.08% TFA in 95% acetonitrile and 5% H_2O). A straight concentration gradient from 0 to 60% buffer B in 64 min was used. The individual peaks were sequenced on an Applied Biosystems model 470 sequenator equipped with on-line phenylthiohydantoin (PTH) analysis using the regular program 03RPTH.

RESULTS

Characterizations of the proband and his family

The proband, K.N., was a 29-year-old male. Both of his parents were born in Kochi, Japan. The family tree showed no instances of marriage between close relatives. In 1984, the patient was diagnosed as having hyperlipidemia, and he was referred to our department. There were no subjective symptoms, the patient was not over-weight, there were no xanthomas of the skin or tendons, and no enlargement of the liver or spleen. The serum total cholesterol was 214–308 mg/dl, while the triglyceride level was in the range of 546–1,568 mg/dl. Figure 1 presents the pedigree of the patient. The patient's mother has hypertension, while the maternal grandmother has hemiplegia due to a cerebral infarction. None of the maternal members was judged as clearly having coronary heart disease.

Analysis of serum lipids and lipoproteins

Table 1 is a compilation of the data on the serum

Fig. 1. Pedigree of the family of the proband.

lipid concentrations for the proband and his kindred. The proband and his mother, maternal grandmother, father, younger brother and younger sister all showed hypertriglyceridemia. Agarose electrophoresis of the proband's serum revealed a broad-beta band (Fig. 2). The serum from the patient's mother gave similar results. Table 2 presents the data on the lipid concentrations in the various lipoprotein fractions of the proband's plasma; the VLDL-cholesterol and IDL fraction were increased and LDL fraction was decreased. The VLDL-cholesterol/VLDL-triglyceride ratio was 0.26.

The results on serum apoprotein concentrations of the proband showed that the levels of apo E, C-II and C-III were increased (Table 3).

The LPL and H-TGL activities in the post-heparin plasma from the proband were 6.2 and 14.6 μ mol free fatty acid per ml/h, respectively (mean $\pm$ SD from 15 normal healthy men: 7.4 ± 1.6 ,

Fig. 2. Agarose gel electrophoretogram of serum from (a) normal subject with phenotype E3/3 and (b) patient KN.

Table 2. Lipid concentrations of lipoprotein fractions from patients KN.

Fraction*	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Phospholipid (mg/dl)
VLDL	91	349	101
IDL	39	88	39
LDL	40	5	26
HDL	23	1	69

* VLDL, $d < 1.006$; IDL, $1.006 < d < 1.019$; LDL, $1.019 < d < 1.063$; HDL, $1.063 < d < 1.210$

19.5 ± 5.7).

Apo E analysis

The pattern of isoelectric focusing of the proband's apo VLDL was investigated. As seen in Fig. 3, the apo E isoforms showed the characteristic double bands (Fig. 3-b). Of these double bands, the

Table 1. Concentrations of serum lipids in the KN family.

Subject	Sex	Age	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Phospholipid (mg/dl)
I-8	F	76	265	282	262
II-1	M	59	240	243	262
II-4	M	55	179	89	184
II-5	F	52	229	117	222
II-6	F	49	225	343	250
II-7	M	46	192	94	203
III-2	M	29	238	732	273
III-3	F	26	206	204	211
III-4	M	21	205	247	230

Table 3. Concentrations of serum apolipoproteins in patient KN.

	Patient (KN)	Normal*
Apo A-I	98.0	138.7 ± 18.5**
Apo A-II	21.5	30.0 ± 5.0
Apo B	73.0	86.3 ± 16.8
Apo C-II	17.4	3.56 ± 1.10
Apo C-III	32.8	8.60 ± 3.26
Apo E	22.4	3.28 ± 1.08

* n=20 (normal healthy men), **mean ± SD

more cationic components precisely coincided with the electrophoretic migration positions of the E3 band and the sialized forms of apo E3 (E3s1 and E3s2 bands) of the control which was an apo E3 homozygote. The more anionic components of each double band (*1, *2 and *3 in Fig. 3) were separated from their cationic component bands by approximately one-half charge.

In the isoelectric focusing performed on apo VLDL which had been digested with neuraminidase, the double bands at the positions of E3s1 and E3s2

Fig. 3. Isoelectric focusing on polyacrylamide gels of (a) apo VLDL from normal subject with phenotype E3/3; (b) apo VLDL, (c) neuraminidase-digested VLDL and (d) cysteamine-modified VLDL from patient KN. The pH range of the ampholytes used was 4–6.

Fig. 4. Isoelectric focusing of polyacrylamide gels of apo VLDL from nine members of the KN kindred.

(a) normal subject with phenotype E3/3; (b) I-8; (c) II-1; (d) II-4; (e) II-5; (f) II-6; (g) II-7; (h) III-2; (i) III-3; (j) III-4. The pH range of the ampholytes used was 4–6.

were both reduced in intensity (Fig. 3-c). It is thus clear that the components in these double bands are the result of sialization of the material in the double bands corresponding to the E3 position. Isoelectric focusing performed after treatment of apo VLDL with cysteamine gave the results shown in Fig. 3-d. Each set of double bands shifted toward the cathode by a single charge unit. This finding indicates that each of the components of the double bands consists of molecules containing one cysteine residue per molecule.

Figure 4 shows the results of isoelectric focusing of the apo VLDL fractions of some of the proband's relatives. The proband's father, younger brother and younger sister show an apo E3/3 pattern, while his mother, maternal grandmother and one maternal uncle have the same double bands as that of the proband. On the basis of these findings, it is concluded that the cationic components of the proband's double bands were inherited from his father and are apo E3, while the anionic components are an apo E variant which were inherited from his mother.

The apo E of the proband was subjected to two-dimensional electrophoresis (Fig. 5). The spot for apo E3 and that of the apo E variant (*1) were separated by approximately one-half charge on the isoelectric focusing axis, while they were in almost the same position on the SDS axis. The SDS migra-

Fig. 5. Two-dimensional gel electrophoretograms of apo VLDL of patient KN (upper) and normal subject with phenotype E3/3 (lower). Only the region of the gel containing apo E is shown. IEF, isoelectric focusing; SDS, sodium dodecyl sulfate electrophoresis.

tion results show that apo E3 and the apo E variant have the same molecular weight.

Figure 6 shows the results of double immunodiffusion of the purified apo E3 and apo E variant from the proband using anti-human apo E goat serum. The precipitin lines with those samples showed identity and fusion with that formed by the control apo E3. Accordingly, the proband's apo E variant was antigenically identical to apo E3.

Sequence analysis of apo E

Figure 7 shows the reverse phase HPLC separation of lysyl endopeptidase fragments of apo E derived from an apo E3 homozygote and the

Fig. 6. Double-immunodiffusion of apo E3 from normal subject (1); apo E variant (2) and apo E3 (3) from patient KN against anti-human apo E goat serum (A).

Fig. 7. Reverse phase HPLC of lysyl endopeptidase digests of apo E from an apo E3 homozygote and apo E-Kochi proband. The digests of 100 μ g apo E were subjected to HPLC under the conditions described in the text.

proband. The fragment L6 from an apo E3 homozygote as a control had the sequence Leu-Arg-Lys. The peak of fragment L6 from the proband was half the height, and a fragment L6' which was not recognized in digestion from apo E3 homozygote, was found in that from the proband. L6 from the proband had the same sequence, Leu-Arg-Lys, as that from E3 homozygote, and L6' showed the sequence Leu-His-Lys.

DISCUSSION

The apo E variant detected by isoelectric focusing of apo VLDL from the proband has not previously been reported. And it was therefore named apo E-Kochi. The phenotype of the proband can be expressed as apo E3/Kochi.

Variants described as E2 (Arg145 → Cys), E2 (Lys146 → Gln) and E2-Christchurch (Arg136 → Ser) have been reported separately from the typical apo E2 (6, 7, 15). However, those variants can not be distinguished from the ordinary E2 in isoelectric focusing. In addition, E3 (Ala99 → Thr, Ala152 → Pro), E3-Leiden and E3 (Cys112 → Arg, Arg142 → Cys) have been reported as variants having the same pI as E3 (12, 14, 16). In addition, E-Bethesda, E-5 (Glu3 → Lys), E1 (Gly127 → Asp, Arg158 → Cys), E-Suita (Glu244 → Lys, Glu245 → Lys) and E1-Harrisburg have been discovered as variants with pI values differing from those of the typical apo E4, E3 and E2 (9–11, 13, 17, 26, 27). There have, however, been no reports to date of any variant showing phenomena similar to these presented by apo E-Kochi, which differs from apo E3 in pI by approximately half a charge and forms distinct double bands with apo E3 on isoelectric focusing.

The isoelectric difference between apo E3 and E-Kochi in the proband was too close. It was difficult to isolate a sufficient amount of apo E-Kochi. So the mixture of apo E3 and E-Kochi was analyzed by peptide sequencing. The fragment L6

from apo E3 homozygote had the sequence Leu-Arg-Lys, and this sequence coincided only with residue 141–143 or 144–146 of apo E3, of which a complete sequence had already been determined (Fig. 8) (12, 28). Because lysyl endopeptidase is essentially specific for peptide bonds on the carboxyl terminal side of lysine residues, the fragment of 141–143 residues which follows histidine of 140 residue, was not produced. So the peptide of L6 was 144–146 residues. The proband had half of the amount of L6 fragment which had the same sequence as apo E3 homozygote, and L6' derived from the proband had the sequence Leu-His-Lys, which has never been recognized within any sequence of apo E reported to date. The results of the current sequence analysis concluded that apo E-Kochi contains histidine as a substituted for arginine at residue 145. Histidine possesses approximately one-half a proton in its side chain ($pK=6.0$) in the vicinity of the pI of apo E3 ($Ca.6.0$). Therefore, if it is assumed that one arginine residue which has one proton in its side chain in apo E3 is replaced by a histidine, the molecular weight would not be changed, but this molecule may theoretically lose one-half a charge and migrate to the position of apo E-Kochi in the isoelectric focusing procedure.

Patients with type III hyperlipoproteinemia show a broad-beta band in agarose electrophoresis, and this type is exhibited by apo E2 homozygotes. This apo E2 shows a markedly reduced affinity for apo B,E receptors (29) and is considered to be involved in the development of hyperlipoproteinemia. Such rare variants as E-Bethesda, E1, E1-Harrisburg, E2 (Arg145 → Cys), E2 (Lys146 → Gln), E2-Christchurch and E3 (Cys112 → Arg, Arg145 → Cys) are also known to show type III or type III-like hyperlipoproteinemia, and most of these apo E variants show reduced affinity for apo B,E receptors (6–9, 11). Therefore, these variants are also suggested to be involved in the development of hyperlipo-

Fig. 8. Amino acid sequence around the apo B,E receptor domain of apo E3. The sequence is taken from McLean et al (12) and Rall et al (28).

* apo E-Kochi.

proteinemia. However, whereas classical type III hyperlipoproteinemia does not develop from an E2 heterozygote, the above-mentioned apo E variants do in fact develop type III hyperlipoproteinemia even though they are heterozygotes. For this reason, it is suspected that the mechanism behind the development of this disease might be slightly different. In the case of the present apo E-Kochi patient, as well, a broad-beta band was shown upon agarose electrophoresis, cholesterol levels in VLDL fraction were increased and LDL levels were decreased, which are all characteristic findings of type III hyperlipoproteinemia (30). However, the proband's VLDL fraction showed a cholesterol/triglyceride ratio of 0.26, which is lower than the standard value of 0.42 reported by Hazzard et al for type III hyperlipoproteinemia (31). Thus, in view of these points, it is perhaps difficult to make a firm conclusive decision as to the phenotype of the present case.

Residue 145 which was replaced by histidine for arginine in apo E-Kochi is contained within the 140–160 residue positions which are important for apo B,E receptor binding (32, 33). Thus, the cause of hyperlipoproteinemia in the proband (III-2), his mother (II-6) and maternal grandmother (I-8) who had apo E-Kochi in their serum, may be related to this apo E variant. But the proband's uncle (II-7) was not affected with hyperlipoproteinemia in spite of the presence of the same variant. For this reason, even if apo E-Kochi plays a part in the etiology, another factor may be necessary for the expression of hyperlipoproteinemia. In order to clarify the relationship between hyperlipoproteinemia and apo E-Kochi, the binding ability of apo E-Kochi to apo B,E receptors and the possibility of other factors must be elucidated in the future.

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