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APOLIPOPROTEIN E5 AND E7 IN APPARENTLY HEALTHY JAPANESE MALES: FREQUENCIES AND RELATION TO PLASMA LIPID LEVELS

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Summary In order to determine the frequencies of apolipoproteins (apo) E5 and E7 and their relation to plasma lipid levels, apo E phenotypes were determined in 608 healthy Japanese male adults by two-dimensional gel electrophoresis. Apo E5 and E7 were observed in 2.8% of the subjects, in addition to the three common apo E isoforms, E2, E3, and E4. Apo E5 was divided into two subtypes based on the migration rate on SDS/PAGE, E5f is the type with faster migration and E5s slower migration. The gene frequencies were: the ε3 allele, 0.841; the ε4 allele, 0.095; the ε2 allele, 0.049; the ε7 allele, 0.009; the ε5 allele encoding apo E5f (the ε5f allele), 0.004; and the ε5 allele encoding apo E5s (the ε5s allele), 0.001. The five individuals with apo E5f and the eleven with apo E7 were heterozygotes and normocholesterolemic. Also plasma apo B and apo E levels were not increased in any subjects with apo E5f or apo E7. The data suggests that apo E5f and E7 are not rare in the Japanese population but that neither apo E5f nor E7 are associated with hypercholesterolemia in most of the heterozygotes.

Key Words apolipoprotein E5, apolipoprotein E7, apolipoprotein E (apo E) lipid level, hypercholesterolemia, allele frequency

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INTRODUCTION

Apolipoprotein E (apo E) is a constituent of chylomicrons, chylomicron remnants, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL). It plays an important role in determining triglyceride-rich lipoprotein catabolism by mediating the cellular uptake of specific lipoproteins such as IDL and chylomicron remnants through an interaction with apo B/E (LDL) receptors and distinct hepatic apo E receptors (Mahley, 1988). It has been shown that the deletion of apo E and an apo E variant which has no binding activity to the receptor, leads to hyperlipoproteinemia (HLP) type III (Utermann *et al.*, 1977; Ghiselli *et al.*, 1981).

Human plasma apo E is composed of 299 amino acid residues and has a calculated molecular weight of 34,200. In plasma there are three common isoforms of apo E (E2, E3, and E4) which differ from each other in their charge by one unit and are detected by isoelectric focusing (IEF). Apo E3 is the most commonly occurring form. The apo E2 isoform, which has an Arg→Cys substitution at position 158, is associated with decreased plasma levels of total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C). It has been shown that apo E2 has a decreased fractional catabolic rate and markedly impaired binding to the apo B/E receptor as compared to apo E3 (Gregg *et al.*, 1981; Weisgraber *et al.*, 1982; Rall *et al.*, 1982), causing type III HLP in some homozygotes. On the other hand, the apo E4 isoform, which has a Cys→Arg substitution at position 112, is associated with elevated levels of plasma TC and a LDL-C, though apo E4 and apo E3 have similar binding properties to the apo B/E receptor (Weisgraber *et al.*, 1982). It has been shown that apo E4 is catabolized more rapidly than apo E3, possibly resulting in the down-regulation of the liver LDL receptor and increased levels of plasma LDL (Utermann *et al.*, 1977, 1984; Ehnholm *et al.*, 1984; Gregg *et al.*, 1986; Utermann, 1987; Ordovas *et al.*, 1987). Also, it has been revealed that apo E4 is a major risk factor for late-onset Alzheimer's disease (Corder *et al.*, 1993; Saunders *et al.*, 1993; Poirier *et al.*, 1993; Noguchi *et al.*, 1993; Yoshizawa *et al.*, 1994).

Other minor isoforms have been detected and designated from apo E1 to apo E7 according to the reverse order of mobility of IEF. Apo E5 and E7 were first detected in Japanese patients with hyperlipidemia (Yamamura *et al.*, 1984a, b). Apo E5 is derived from E3 by a Glu→Lys substitution at position 3 [(Glu_3→Lys)] (Tajima *et al.*, 1988; Maeda *et al.*, 1989a). Since this type of apo E5 migrates faster than apo E3 on SDS/PAGE, we have tentatively designated it apo E5f. It was recently reported that the receptor binding activity of apo E5f is about twice that of normal apo E (Dong *et al.*, 1990). Apo E5 with the same migration as apo E3 on SDS/PAGE has also been found among Caucasians in the United States (Ordovas *et al.*, 1987). We have tentatively designated it as apo E5s. Further-

more, a novel apo E5 derived from E3 by a Glu→Lys substitution at position 13 [E5(Glu_{13}→Lys)] and another novel apo E5 derived from E4 by a Pro→Arg substitution at position 84 [E5(Pro_{84}→Arg, Cys_{112}→Arg)] were discovered in two French-Canadian subjects (Mailly *et al.*, 1991) and in a subject of European descent (Wardell *et al.*, 1991), respectively. On the other hand, Apo E7 is derived from E3 by a Glu→Lys substitution at positions 244 and 245 [E7($Glu_{244,245}$→Lys)] (Maeda *et al.*, 1989b; Tajima *et al.*, 1989). Thus far, apo E7 has been detected only among Japanese (Yamamura *et al.*, 1984a, b; Tsuchiya *et al.*, 1985). As for apo E5 and E7, there are no reports regarding their frequencies and relations with plasma lipid levels in general populations.

The following describes the frequencies of apo E5 and E7 and their relations to the plasma levels of TC, LDL-C, HDL-cholesterol (HDL-C), triglyceride (TG), apo B, and apo E in 608 apparently healthy Japanese male adults.

MATERIALS AND METHODS

Subjects were 608 randomly selected apparently healthy Japanese males aged 24 to 65 with a mean age of 47.6 years, who visited the health care center of Kudanzaka hospital in Tokyo for their annual medical examinations. Males with a history of surgery involving the digestive system or those who had been found to have abnormalities of the liver and/or renal functions, or diabetes mellitus at the time of blood sampling were excluded from the investigation. Blood samples were taken after an overnight fast.

Apo E phenotypes were determined by two-dimensional gel electrophoresis, according to the method of O'Farrell (1975) with a minor modification (Hamaguchi *et al.*, 1982). Briefly, 10 μl of VLDL-rich fraction was separated from 180 μl of serum by ultracentrifugation for 38 min at 100,000 rpm using a Beckman TLA-100 rotor. Ampholine mixtures of 1.4% pH range 5–8 and 0.6% pH range 3–10 were used in the first-dimensional disc gel. After the two-dimensional gel electrophoresis, the polypeptides were visualized on a slab gel using the silver stain technique of Merril *et al.* (1981). The apo E spots on the slab gels were confirmed by immuno-blotting (Towbin *et al.*, 1979), using goat anti-human apo E (Dai-ichi, Tokyo) and peroxidase-conjugated rabbit anti-goat IgG (Cappel Laboratories Inc., Malvern, Pa.). This method makes it possible to detect apo E variants with basic pI, such as apo E7.

TC and TG levels were assayed enzymatically. HDL-C levels were measured by the heparin-Ca^{2+}-Ni^{2+} precipitation procedure (Noma *et al.*, 1979). The concentration of LDL-C was calculated according to Friedewald's formula (Friedewald *et al.*, 1972). Apo B and apo E levels were assayed by a single radial immunodiffusion analysis (SRID), (APOE, APOB PLATE, Dai-ichi, Tokyo).

Allele frequencies were estimated by the gene counting method. In-pair differences between apo E phenotypic groups were estimated using Scheffe's procedure

(parametric test) as well as the Mann-Whitney U test (nonparametric test). Prior to the statistical analyses, the lipid and lipoprotein traits were adjusted by multiple linear regression for variation in age, height, and weight. For analysis of plasma TG which did not have normal distributions, logarithmic transformation was performed.

RESULTS

The apo E phenotype distribution and apo E allele frequencies in 608 apparently healthy Japanese males are presented in Table 1. The distribution of the different apo E phenotypes were on the Hardy-Weinberg equilibrium ($\chi^2=6.73$, $df=10$, not significant). The frequencies of the ε2 allele (0.049) and the ε4 allele (0.095) were significantly decreased and that of the ε3 allele (0.841) was significantly increased compared with those in most Caucasian populations (Wardell *et al.*, 1982; Menzel *et al.*, 1983; Utermann *et al.*, 1984; Ordovas *et al.*, 1987; Smit *et al.*, 1988). The frequencies of the three common alleles in the Japanese popula-

Table 1. Apo E phenotype and allele frequencies in apparently healthy Japanese males.

Apo E phenotype	Number (%)
3/3	432 (71.1)
3/4	96 (15.8)
2/3	50 (8.2)
2/4	9 (1.5)
3/7	9 (1.5)
4/4	4 (0.7)
3/5f	4 (0.7)
4/7	2 (0.3)
2/5f	1 (0.2)
4/5s	1 (0.2)
2/2	0 (0.0)
Total	608
Apo E allele	**Frequencies**
ε2	0.049
ε3	0.841
ε4	0.095
ε5f	0.004
ε5s	0.001
ε7	0.009

tions were not significantly different from those reported by Yamamura *et al.* 1984a, b), Tsuchiya *et al.* (1985), and Eto *et al.* (1985, 1986). It is noteworthy that 6 (1.0%) and 11 (1.8%) out of the 608 were found to have apo E5 and apo E7, respectively, in a heterozygous state.

Figure 1 shows examples of the two-dimensional gel electrophoresis patterns

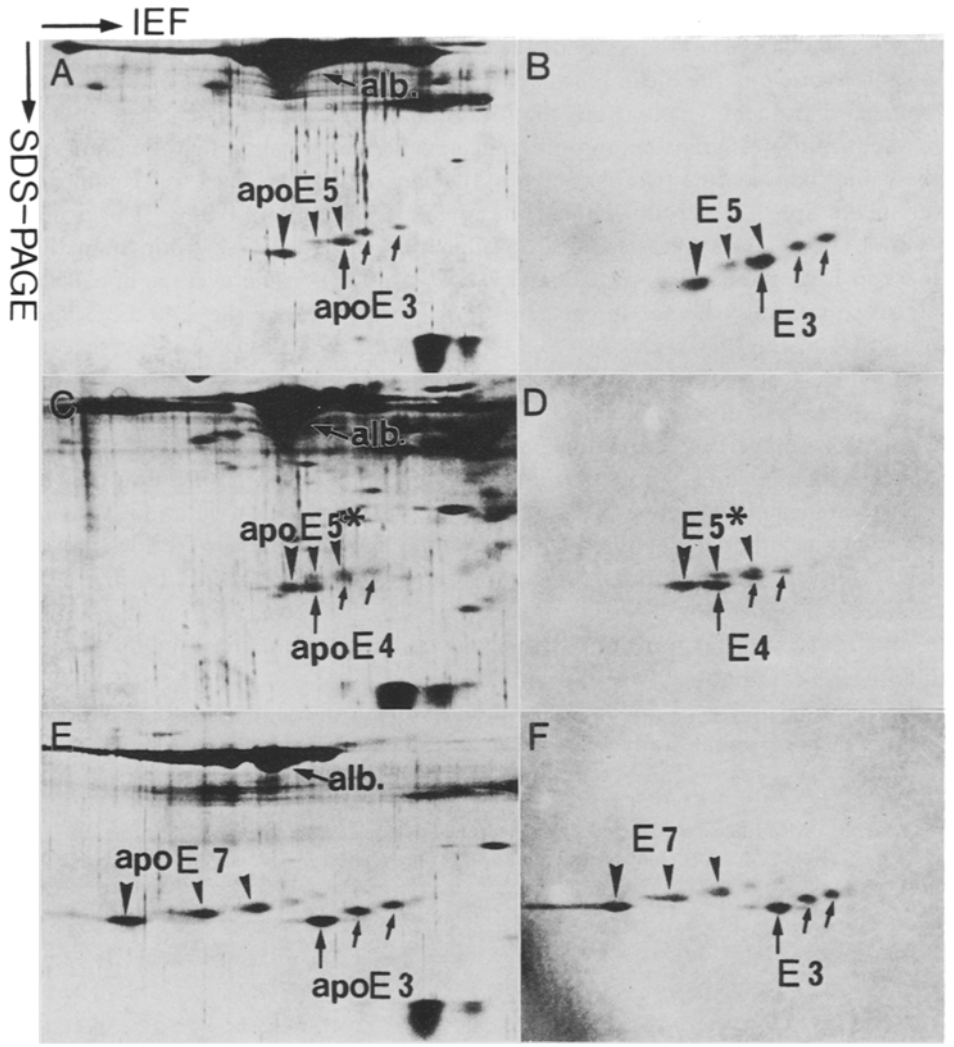

Fig. 1. Two-dimensional gel electrophoresis patterns of the apo E5f, E5s, and E7 phenotypes. A, silver staining of apo E3/5f; B, immuno-blotting of apo E3/5f; C, silver staining of apo E4/5s; D, immuno-blotting of apo E4/5s; E, silver staining of apo E3/7; F, immuno-blotting of apo E3/7. Isoelectric focusing was from left to right and molecular weight separation was from top to bottom. Albumin is shown as "alb."

of apo E3/5f, apo E4/5s, and apo E3/7 phenotypes, together with the data on the identification of the apo E spot on the gel by immuno-blotting. The frequency of the apo E7 allele was 0.009. As for apo E5, two different isoforms were found: apo E5f with a faster migration than apo E3f (Fig. 1, a and b) on SDS/PAGE, which was detected in five subjects; and apo E5s with the same migration as apo E3 on SDS/PAGE (Fig. 1, c and d), which was observed in one subject with apo E4/5. Most of the apo E5 detected among the Japanese was apo E5f, and its allele frequency was 0.004. The frequency of the E5s allele was 0.001.

Table 2 presents the mean plasma TC, LDL-C, HDL-C, and TG, levels among different apo E phenotypes. Both the total cholesterol and LDL cholesterol levels were significantly different among different apo E phenotypes. In the comparison of the values between the two phenotypes, the average LDL-C level was significantly lower in the apo E2/3 group than in the apo E3/3 group ($p<0.01$). The average TC and LDL-C levels were significantly higher in the apo E3/4 group than those in the apo E3/3 group (TC, $p<0.001$; LDL-C, $p<0.01$). The average apo B level was also significantly higher in the apo E3/4 group than in the apo E3/3 group (mean$\pm$SD, 82.9 ± 20.6 mg/dl *vs.* 75.8 ± 16.1 mg/dl, $p<0.001$). The average apo B level tended to be lower in the apo E2/3 group than the apo E3/3 group (mean$\pm$SD, 70.5 ± 17.1 mg/dl *vs.* 75.8 ± 16.1 mg/dl) but the difference was not significant. The results with the three common phenotypes detected in our samples are in accordance with the data reported thus far (Utermann *et al.*, 1979; Bouthillier *et al.*, 1983; Sing and Davignon, 1985; Eto *et al.*, 1986; Boerwinkle and Utermann, 1988). On the other hand, the average TC and LDL-C levels were lower in the subjects with apo E3/5f and 3/7 than those in the apo E3/3 group, but the differences were not significant. Also the average apo B and apo E levels in the subjects with apo E3/5f and 3/7 were not significantly different from those in the subjects with apo E3/3 (data not shown).

The plasma lipid and apolipoprotein levels of all individuals with apo E5 and E7 found in the present study are shown in Table 3. The only male subject with

Table 2. Mean plasma cholesterol, triglyceride, and HDL-cholesterol levels (in mg/dl) among different apo E phenotypes.

Phenotype (n)		TC	TG	HDL-C	LDL-C
2/3	(50)	181.5±32.5	136.0±89.7	47.7±13.2	106.6±33.3
3/3	(432)	189.6±31.4	118.6±59.1	47.5±12.5	118.4±28.6
3/4	(96)	200.7±32.0	136.4±70.7	47.1±15.6	126.3±31.1
3/5f	(4)	182.0±30.3	87.8±24.1	50.6±5.8	113.9±26.0
3/7	(9)	185.3±28.0	149.2±61.9	51.6±14.0	103.9±23.2
Significance[a]		$p<0.01$	not significant	not significant	$p<0.001$

[a] Level of significance estimated by one-way analysis of variance.

Table 3. Individual data on subjects with apo E5 and apo E7.

Apo E phenotype	Age (year)	TC	TG	HDL-C (mg/dl)	LDL-C	Apo B	Apo E
2/5f	37	178	188	39.6	100.7	ND	ND
3/5f	41	143	66	49.2	80.6	52	2.8
3/5f	51	183	120	44.3	114.7	73	3.0
3/5f	43	217	73	58.3	144.1	108	3.9
3/5f	36	185	92	50.6	116.0	83	3.5
4/5s	59	240	98	43.3	177.1	ND	ND
3/7	52	167	139	46.9	92.3	72	3.0
3/7	40	202	88	58.6	125.8	72	3.5
3/7	50	135	136	41.9	65.9	57	3.0
3/7	43	204	164	44.6	126.6	ND	ND
3/7	59	196	128	46.5	123.9	83	3.2
3/7	39	158	145	39.6	89.4	72	3.2
2/7	53	228	253	47.1	130.3	104	4.7
3/7	48	195	232	53.3	95.3	88	4.9
3/7	36	183	58	85.8	85.6	52	4.4
4/7	40	168	69	38.4	115.8	78	2.6
4/7	44	202	91	41.5	142.3	83	3.3

The average apo B and apo E levels were 75.8±16.1 mg/dl and 3.8±0.8 mg/dl, respectively, in randomly selected 283 subjects with apo E3/3. ND, not determined.

apo E4/E5s was hypercholesterolemic (TC, 240 mg/dl; LDL-C, 177.1 mg/dl). All individuals with apo E5f or E7 were heterozygotes and their TC levels were less than 230 mg/dl, while 70 out of 608 subjects (11.5%) in the present study had cholesterol levels above 230 mg/dl. Plasma apo B and apo E levels were also within normal ranges in all subjects with apo E5f or E7.

DISCUSSION

The present study confirms racial differences related to three common apo E allele frequencies between Japanese and Caucasian populations (Eto *et al.*, 1986; Hamaguchi *et al.*, 1992). Even if apo E5f and apo E7 were included, apo E variants are less frequent in the Japanese when compared with Caucasian populations. Apo E5f, E5s, and E7, however, were found in 0.9%, 0.2%, and 1.8%, respectively, of the subjects, totaling nearly 3%. Following the first detection of apo E5f and apo E7 in Japanese patients with hyperlipidemia (Yamamura *et al.*, 1984a, b), they were observed in apparently healthy Japanese subjects (Tsuchiya *et al.*, 1985; Yanagi *et al.*, 1990). The data obtained in the present study suggests that the occurrence

of subjects with apo E5 or E7 is not rare and that apo E7 is more common than apo E5 in the Japanese population. The reason why apo E7 is observed in Japanese at a frequency near the polymorphic level remains to be elucidated. One possibility is that a founder effect occurred in Japanese. The other possibility is a difference in the method used to analyze apo E phenotypes. In the present study, a method to detect apo E variants with basic PI, such as apo E7, was used in isoelectric focusing, while conventional isoelectric focusing for the analysis of apo E phenotypes might not detect of apo E7. Further population studies using appropriate methods to detect apo E7 are needed to determine whether apo E7 is present only in Japanese.

The study also shows that all five subjects with apo E5f and all eleven subjects with apo E7 were normocholesterolemic and both groups of subjects with the apo E3/5f genotype or E3/7 genotype had rather decreased average plasma TC and LDL-C levels (Tables 2 and 3). Apo E5f and apo E7 were originally found in patients with hypercholesterolemia (Yamamura *et al.*, 1984a, b). The heterozygote for apo E7 was observed in 2 out of 58 lipid clinic patients (3.4%) and 2 out of 69 CCU patients (2.9%), while the heterozygote for apo E5f was found in 2 out of 58 lipid clinic patients (3.4%) and 1 out of 69 CCU patients (1.4%) (Yamamura *et al.*, 1984b). It has been proposed that apo E5 and E7 might increase plasma levels of TC and LDL-C since the average plasma cholesterol levels increase as the net charge of the apo E molecule increases from apo E2 to E4 and apo E5f has two and E7 has four additional positive net charges (Tajima *et al.*, 1989). Individuals with apo E5f and apo E7, however, were also observed among normocholesterolemic populations (Tsuchiya *et al.*, 1985; Yanagi *et al.*, 1990), implying that individuals with apo E5 or those with apo E7 are not always hypercholesterolemic. The results of the present study further indicate that most of the individuals with apo E5f and those with apo E7 are normocholesterolemic at least in the heterozygous state. The data on plasma apo B and apo E levels also support this interpretation. Furthermore, the frequencies of heterozygotes for apo E7 (1.8%) and for apo E5f (0.9%) observed in our population were not significantly different from those found in lipid clinic patients by Yamamura *et al.* (1984b). As for apo E5s, two subjects with apo E3/5s found in the USA (Ordovas *et al.*, 1987) and one with apo E4/5s observed in the present study had elevated TC and LDL-C levels. Whether apo E5s has an effect of elevating TC and LDL-C levels remains to be elucidated.

In conclusion, the results of the present study suggest that apo E5f and E7 are not rare in the Japanese population but, do not cause hypercholesterolemia in most of the heterozygotes.

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