Determination of Apolipoprotein E in High Density Lipoprotein Fraction by Immunofixation Method and Turbidimetric Immunoassay After Precipitation

Yasuyuki Okamoto1, Hiroyuki Tsujii1, Yoshimasa Haga2, Shinobu Tanaka2, and Hiroshi Nakano1

1Department of Clinico-Laboratory Diagnostics and 2Division of Central Clinical Laboratory, Nara Medical University, Nara, Japan.

Apolipoprotein E (apoE) in high density lipoprotein (HDL) fraction (HDL-fr) was determined by the immunofixation method and turbidimetric immunoassay (TIA) after precipitation with phosphotungstic acid/MgCl2 in normolipidemic control subjects and patients with type IV hyperlipemia and hyper HDL-cholesterolemia. Immunofixation assay revealed two major bands of apoE in whole serum: one in the \( \alpha \)-area and the other in the pre\( \beta \)-area. ApoE in the \( \alpha \)-area (\( \alpha \)-apoE) was identical to \( \alpha \)-apoE in HDL-fr separated by ultracentrifugation but not to \( \alpha \)-apoE in HDL-fr separated by precipitation (pHDL-fr). \( \alpha \)-ApoE in pHDL-fr lacked the slower area of the band. Agarose column chromatography and gradient gel electrophoresis indicated that \( \alpha \)-apoE belongs to early fractionated HDL, and that the precipitable \( \alpha \)-apoE belongs to the higher molecular size HDL. \( \alpha \)-ApoE (%) estimated by immunofixation showed a strong positive correlation with apoE (%) in pHDL-fr. ApoE (%) in pHDL-fr was higher in case of hyper HDL-cholesterolemia and lower in type IV hyperlipemia than in controls, and was inversely correlated with serum triglycerides (TG) and positively with HDL-cholesterol (especially HDL2-cholesterol) in these subjects. It is suggested that the variation of apoE in pHDL-fr depends on the level of HDL2. Also, it may be suggested that apoE in pHDL-fr and precipitable \( \alpha \)-apoE belong to low and high molecular HDL2, respectively, and that apoE content in these particles is correlated with HDL2.

Key words: Phosphotungstic acid/MgCl2, Apolipoprotein A-I, Lipoprotein lipase.

Apolipoprotein E (apoE) is widely distributed among plasma lipoproteins and mainly functions as a ligand in the receptor-mediated catabolism of lipoproteins which contain apoE (especially remnants) (1, 2). Most apoE in plasma is associated with very low density lipoprotein (VLDL) and high density lipoprotein (HDL) fractions, and is transferable between them (3, 4). In HDL fractions containing a large amount of apoE, it is usually associated with HDLc or HDL1 which are known to be increased by high cholesterol diets (5) and in patients with homozygous familial hypercholesterolemia (6) and hyperalphalipoproteinemia with cholesteryl ester transfer protein (CETP) deficiency (7). It is reported that increased apoE-rich HDL is characteristic of hyper HDL-cholesterolemia with CETP-deficiency (7). However, little is known about the nature and clinical significance of lipoproteins which contain apoE in the HDL fraction (HDL-fr). In this study, we determined apoE in HDL-fr by the immunofixation method after agarose gel electrophoresis and turbidimetric immunoassay (TIA) after precipitation with phosphotungstic acid/MgCl2 in normolipidemic control subjects, and patients with type IV hyperlipemia and hyper HDL-cholesterolemia.

Subjects and Methods

Serum samples were obtained from normolipidemic control subjects, and patients with type IV hyperlipemia...
Okamoto et al.

Table 1. Characteristics of subjects enrolled in the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Hyper HDL-cholesterolemia</th>
<th>Type IV hyperlipemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Number of cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean BMI (Kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol user (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Serum lipids:
- **Tch (mg/dl)**: 174.3 ± 26.01, 170.9 ± 25.05, 194.0 ± 22.49, 193.4 ± 27.2, 169.7 ± 12.94, 169.5 ± 36.24
- **TG (mg/dl)**: 96.2 ± 36.28, 69.1 ± 27.9, 67.8 ± 20.23, 83.6 ± 33.55, 507.5 ± 127.0, 278.3 ± 91.25
- **HDL-ch (mg/dl)**: 51.7 ± 9.23, 57.4 ± 8.83, 85.0 ± 7.78, 85.1 ± 7.88, 28.2 ± 8.57, 41.5 ± 19.91
- **LDL-ch (mg/dl)**: 103.4 ± 20.78, 99.7 ± 21.18, 95.4 ± 17.41, 91.6 ± 22.06, n.d., n.d.

Serum apolipoproteins:
- **ApoA-I (mg/dl)**: 133.7 ± 19.64, 137.4 ± 17.85, 180.2 ± 24.79, 187.7 ± 24.96, 120.0 ± 23.14, 140.5 ± 50.63
- **ApoB (mg/dl)**: 74.6 ± 14.92, 67.2 ± 13.85, 65.2 ± 7.01, 67.8 ± 13.57, 110.2 ± 14.97, 92.3 ± 14.03
- **ApoE (mg/dl)**: 4.4 ± 0.88, 4.2 ± 1.35, 4.8 ± 1.16, 5.0 ± 1.67, 9.5 ± 2.08, 7.6 ± 1.49
- **ApoE in pHDL-fr (%)**: 9.1 ± 4.25, 12.0 ± 6.91, 18.9 ± 5.52, 18.9 ± 8.37, 2.5 ± 0.89, 3.9 ± 0.83

Data are expressed as means ± SD. n.d.: not detected
*; higher (p < 0.05), **; higher (p < 0.01), †; lower (p < 0.01), compared with value of same sex in control.
BMI; body mass index (body weight/height²). Other abbreviations used are described in the text.

(without any complications) and hyper HDL-cholesterolemia (including pregnant women and healthy men) after an overnight fast. Informed consent was obtained from all the subjects (aged 20 to 60 yrs) prior to enrollment in the study. Serum HDL-cholesterol (HDL-ch) levels in subjects with hyper HDL-cholesterolemia were all higher than 70 mg/dl, which is the upper limit of the reference range in our laboratory. Characteristics of the subjects are shown in Table 1. In these subjects, mean (and range) daily intake of total fat, calories from fat, and cholesterol which were estimated from their own reports on diet for the week before enrollment in the study were 66.8 g (32.2-109.5 g), 25.5% (17.1-35.1%), and 320.7 mg (195.2 - 488 mg), respectively. The polyunsaturated/saturated fat ratio was 0.58 (0.26-1.12). All alcohol users consumed less than 40 g ethanol per day, and smokers consumed fewer than 15 cigarettes per day. No subject was taking any medication. No significant differences were found in mean age, mean body mass index (BMI), proportion of alcohol users or smokers between control and patients with hyper HDL-cholesterolemia or type IV hyperlipemia except for a difference in the proportion of alcohol users between the male control and male subjects with hyper HDL-cholesterolemia.

**Assays**

Samples were used for routine laboratory tests, including assays of total cholesterol (Tch), triglycerides (TG), HDL-ch, and apolipoproteins A-I (apoA-I), B (apoB) and E. Serum levels of apolipoproteins were assayed by TIA in a Hitachi 7150 automatic analyzer with a reagent kit commercially available in Japan (ApoAuto “Daiichi” from Daiichi Pure Chemicals Co. Ltd., Tokyo). Purities and specificities of anti-sera in the kit were evaluated in a previous study by Sakai et al. (8). HDL-ch was determined after precipitation of low density lipoprotein (LDL) and VLDL with phosphotungstic acid and MgCl₂ (9). ApoE in HDL-fr separated by the precipitation procedure (pHDL-fr) was assayed by TIA with reference to the method of Funke and Assmann (10) and expressed as % of total serum apoE. The reproducibility and linearity of this assay were adequate. For precipitation, 200 μl of the precipitating reagent (containing 10 mg/ml of phosphotungstic acid and 40 mM MgCl₂) was added to 200 μl of serum. The mixture was incubated for 10 min at room temperature and centrifuged for 10 min at 3,000 rpm. The supernatant was saved for assay of cholesterol and apoE, and immunofixation. The precipitate was redissolved in 200 μl of 0.2 M NaCl containing 1 mM EDTA (pH 7.0) and saved for immunofixation. LDL cholesterol (LDL-ch) level was calculated using the formula described by Fridewald et al. (11). Serum lipids, apolipoproteins and apoE (%) in pHDL-fr in the subjects are shown in Table 1.

Electrophoresis for immunofixation was performed with the agarose gel film Universal Gel/8 (1% agarose in 65 mM barbital buffer pH 8.6, purchased from Ciba Corning Diagnostics Corp., Palo Alto, USA) for 45 min under constant voltage (90 V). For immunofixation, the agarose gel was covered with anti-apolipoprotein antiserum-absorbed cellulose acetate film for one hour at room temperature, washed with saline for 24h and stained with Coomassie Brilliant Blue R-250. The amount of serum used
for apoE immunofixation was either 3 µl or 6 µl. ApoA-I- and apoB-free serum was prepared with the mixture of anti-apoA-I and anti-apoB monoclonal antibody-conjugated Sepharose 4B (kindly provided by Japan Immunoresearch Laboratories Co. Ltd., Takasaki). Proportion of apoE distribution was estimated by densitometrical scanning with calibration by scanning the background stain. The anti-apoE polyclonal anti-serum used for immunofixation was obtained from Chemicon International, Inc., Temecula, USA. The specificity of anti-serum used was

Fig. 1. Immunofixation of apolipoprotein E. Lanes 1-3; a male control subject (lanes 1, 2 and 3 are apoE in whole serum, pHDL-fr and the precipitate, respectively), 4-6; a male subject with type IV hyperlipemia (lanes 4, 5 and 6 are apoE in whole serum, pHDL-fr and the precipitate, respectively), 7-9; a female subject with hyper HDL-cholesterolemia (lanes 7, 8 and 9 are apoE in whole serum, pHDL-fr and the precipitate, respectively). Lanes 10-13; apoE in lipoprotein fractions separated by ultracentrifugation from fresh pooled serum (lanes 10, 11, 12 and 13 are apoE in HDL, LDL, VLDL and LPDS, respectively). The volume of sample used for electrophoresis was 3 µl of whole serum and the redissolved precipitate, and 6 µl of the supernatant after precipitation. Abbreviations used are described in the text.
tested by ouchterlony gel diffusion.

Fractionation of lipoproteins was performed by preparative ultracentrifugation and gel chromatography. HDL (d=1.063-1.200), LDL (d=1.006-1.063), VLDL (d<1.006) and lipoprotein-deficient sera (LPDS, d>1.200) were isolated from fresh pooled-serum by the usual ultracentrifugation method (12). For gel chromatography, 10 ml of whole serum obtained from a woman with hyper HDL-cholesterolemia was applied to a 2.6×100 cm column of 2% agarose (Biogel A-50 m 100-200 mesh, BioRad Laboratories, Richmond, CA) in 0.2 M NaCl containing 1 mM EDTA and 0.01% sodium azide (pH 7.0) and collected in 3.5–4 ml fractions. The fractionated samples and the samples after precipitation were applied to electrophoresis on a 2.15% linear gradient polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane for immunoblotting with anti-apoE anti-serum.

ApoE phenotype was ascertained in the subjects with hyper HDL-cholesterolemia by isoelectric focusing with 5% polyacrylamide gel containing 2% Ampholine (pH 3.5–10) and 8 M urea. The serum sample was incubated with neuraminidase solution, delipidated with ethanol and diethyl ether, and solubilized in 10 mM Tris-HCl containing 8 M urea and 10 mM dithiothreitol (pH 8.6). After electrophoresis, the separated sample was transferred to a PVDF membrane for immunoblotting with anti-apoE

![Figure 2](image)

**Fig. 2.** Cholesterol, apolipoprotein A-I, A-II and E distribution, and immunofixation of apolipoprotein E in serum fractionated by agarose column chromatography.  ●—●: cholest

**Fig. 2.** Cholesterol, apolipoprotein A-I, A-II and E distribution, and immunofixation of apolipoprotein E in serum fractionated by agarose column chromatography.  ●—●: cholesterol, ○—○: apolipoprotein A-I, □—□: apolipoprotein A-II, ×—×: apolipoprotein E. Each plot of apolipoprotein E represents the ten times-value. Serum was obtained from a woman with hyper HDL-cholesterolemia, applied to a 2.6×100 cm column of 2% agarose in 0.2 M NaCl containing 1 mM EDTA (pH 7.0), and collected in 3.5–4 ml fractions. Two peaks of cholesterol are associated with LDL and HDL fractions, respectively.
Determination of ApoE in HDL by Immunofixation and TIA

Immobilon PVDF transfer membrane was purchased from Daiichi Pure Chemicals Co. Ltd., Tokyo. Ampholine was purchased from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. Neuraminidase type V was obtained from Sigma Chemical Company, St. Louis, MO. All other chemicals were purchased from Nacalai Tesque Co., Kyoto.

Statistical methods
Data are expressed as means ± SD. Statistical comparisons were done with Student's unpaired two-tailed t-test and chi-square test. Significance of correlation coefficients was also determined by Student's t-test after t-value calculation with the formula t² = (n-2) × r²/(1-r²).

Results
As shown in Fig. 1, immunofixation assay demonstrated two major distinct bands of apoE in whole serum, one in the α-area and the other in the preβ-area (lanes 1, 4 and 7). Mobilities of apoE in the α-area (α-apoE) and apoE in the preβ-area were coincident with those of apoE in HDL-fr (lane 10) and apoE in the VLDL fraction (lane 12) separated by the ultracentrifugation procedure, respectively. Moreover, α-apoE included two different peaks (clearly appearing in lanes 1 and 7). α-apoE from pHDL-fr lacked the slower area of the band (lanes 2, 5 and 8), which was recovered in the redissolved precipitate (lanes 3, 6 and 9). In the cases with normolipidemia, type IV hyperlipemia and hyper HDL-cholesterolemia shown in Fig. 1, the amount of α-apoE in the supernatant seemed to be positively correlated with that in the precipitate.

In four control subjects (two men and two women), levels of Tch and TG in pHDL-fr were compared with those in HDL-fr separated by ultracentrifugation. Total cholesterol and TG levels in pHDL-fr were slightly lower than those in HDL-fr separated by ultracentrifugation. Differences in Tch levels between these fractions ranged from 0.2 to 8.6 mg/dl, and those in TG levels ranged from 2.3 to 10.8 mg/dl. Also, immunofixation of apoE in the serum, from which apoA-I and apoB were removed with

![Fig. 3](image-url)  
**Fig. 3.** Immunoblotting of apolipoprotein E after gradient gel electrophoresis of the peak fraction of apolipoprotein E in fractionated HDL on an agarose chromatogram (the fraction number 79 in Fig. 2). The sample was used as native and after precipitation. W; whole sample, S; supernatant, P; precipitate.

![Fig. 4](image-url)  
**Fig. 4.** Correlations between apolipoprotein E(%) in HDL fraction separated by precipitation, and α-migrating apolipoprotein E(%) determined by immunofixation (A), triglycerides (B) and HDL cholesterol (C). Abbreviations used in figures are described in the text.
anti-apoA-I and anti-apoB monoclonal antibody-conjugated Sepharose 4B, did not show any apoE band (data not shown).

Fig. 2 shows an agarose column chromatogram of a case with hyper HDL-cholesterolemia. Two cholesterol peaks are associated with LDL and HDL fractions, respectively. The peak fraction of apoE in HDL was eluted earlier than the peak fractions of apoA-I and apoA-II. Immunofixation of apoE in HDL-fr separated by gel chromatography revealed that apoE in early-fractionated HDL was similar to α-apoE in whole serum or HDL-fr separated by ultracentrifugation, and apoE in late-fractionated HDL was similar to α-apoE in pHDL-fr. Then, the peak fraction of apoE in HDL was applied to gradient gel electrophoresis with immunoblotting of apoE after precipitation with phosphotungstic acid/MgCl₂ (Fig. 3). Particles containing apoE in the precipitate showed higher molecular size than those in the supernatant. ApoB was not found in this fraction.

ApoE in pHDL-fr is α-apoE in the supernatant. As shown in Table 1, apoE (%) in pHDL-fr was higher in male and female subjects with hyper HDL-cholesterolemia, and lower in male and female patients with type IV hyperlipemia than in control subjects. Correlations between apoE (%) in pHDL-fr, and α-apoE (%) estimated by densitometry of immunofixation, TG and HDL-ch in all the subjects are shown in Fig. 4. There was a strong positive correlation between apoE (%) in pHDL-fr and α-apoE (%) (r=0.899, p<0.01, Fig. 4A). ApoE (%) in pHDL-fr was negatively correlated with TG (r=−0.555, p<0.01, Fig. 4B), and positively with HDL-ch (r=0.611, p<0.01, Fig. 4C). However, apoE (%) in pHDL-fr varied widely in contrast with HDL-ch levels in the subjects with normal and high HDL-ch levels. Correlations between apoE (%) in pHDL-fr and cholesterol in HDL subfractions were determined in ten hyper HDL-cholesterolemic subjects. ApoE (%) in pHDL-fr showed a significant positive correlation with HDL₂-ch (r=0.736, p<0.05), but not with HDL₃-ch (r=0.153).

Three apoE phenotypes (one man and two women with E4/3, three men and seven women with E3/3, and one man and four women with E3/2) were found in our subjects with hyper HDL-cholesterolemia. ApoE (%) in pHDL-fr in the E4/3 group, the E3/3 group, and the E3/2 group were 24.3±10.97%, 18.0±3.82%, and 17.5±11.01%, respectively. The differences in apoE (%) in pHDL-fr among these groups were not significant.

Discussion

Previous studies on the distribution of apoE among lipoproteins in normal human plasma have shown that all the apoE is present in the form of complexes that contain apoA-I or apoB (4, 13). These results were obtained from studies using gel filtration and immunoaffinity chromatography (except for the ultracentrifugation method, which reportedly produces lipoprotein-unassociated apoE by artificial dissociation) (4). The complexes of apoE with apoA-I are associated with apoE-containing HDL, which is reported to comprise 74.5% of all apoE in plasma by gel filtration (13) and 52.8% by affinity chromatography (4).

In this study, we showed that apoE in the α-area of electrophoresis using whole serum was distributed between two different subspecies of α-lipoprotein such as the particles retained in pHDL-fr and the particles precipitatable with phosphotungstic acid/MgCl₂. Both of these particles belong to HDL-fr separated by ultracentrifugation. The observations from gel chromatography and gradient gel electrophoresis indicated that α-apoE belongs to early fractionated HDL, and that the precipitable particles belong to higher molecular size apoE-containing HDL than the particles retained in pHDL-fr. The precipitable particles are also suspected to have a few milligrams of Tch and TG, and be associated with apoA-I, because apoA-I- and apoB-deficient serum did not show α-apoE, and apoB was not found in the fractionated apoE-containing HDL.

ApoE in the precipitable particles had a relative slow mobility on the α-area, and seemed to be correlated with apoE content in the particles retained in pHDL-fr, since a strong positive correlation between apoE in pHDL-fr and α-apoE was suggested (Fig. 1 and Table 1). This possible correlation in apoE content implies the existence of a close interaction between these particles.

It has been reported that post-heparin lipolysis causes the transfer of apoE from TG-rich lipoproteins to HDL (14). Another report describes the transfer of apolipoprotein C-II from VLDL to HDL (especially to HDL₂) with a positive correlation between LPL activity and plasma HDL-ch (especially HDL₂-ch but not HDL₃-ch) in familial hyperphalipoproteinemia and normal subjects (15). The observations from the present clinical study also indicate that the distribution of apoE in pHDL-fr could be affected by the negative correlation between TG and HDL-ch levels in blood, though apoE in pHDL-fr varied widely in contrast with HDL-ch levels in normo- and hyper HDL-cholesterolemic subjects. However, apoE in pHDL-fr showed a significant positive correlation with HDL₂-ch, but not with HDL₃-ch. The inverse correlation between TG and HDL-ch, and apoE distribution in HDL is considered to be involved in LPL-mediated hydrolysis of TG-rich lipoproteins followed by transfer of degraded particles to HDL₂.

ApoE phenotype is also known to affect the apoE distribution among lipoproteins. There is a report that apoE3 is likely to be distributed in HDL, whereas apoE4 is not (16). Differential regulation of hepatic lipase by apoE phenotype may play a role in the apoE distribution (17). However, in our subjects with hyper HDL-cholesterolemia which showed widely varied levels of apoE in pHDL-fr, apoE phenotype was not significantly related with apoE in pHDL-fr, though the effect of infrequent apoE phenotype...
Determination of ApoE in HDL by Immunofixation and TIA

groups such as E4/4 or E2/2 is still unknown. Thus, it is suggested that the variation of apoE in pHDL-fr mainly depends on the variation of HDL2. Also, it may be suggested that apoE in pHDL-fr and precipitatable α-apoE belong to low and high molecular HDL2, respectively, and that there is a correlation in apoE content between these particles. In conclusion, determination of apoE in pHDL-fr may be a simple clinical method to evaluate HDL2 metabolism.

References