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〈Original Article〉

Reactivity of a detergent, polyoxyethylene derivative, with high density lipoprotein subclass

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Summary A detergent, polyoxyethylene derivative (POED), is often used to measure cholesterol in HDL. This is done because the detergent acts specifically on various lipoproteins, makes lipoproteins soluble, and prevents enzymatic reactions. However, there are indications that measurements are affected by the reactivity of the detergent against HDL-subfractions. We then investigated the characteristics of POED in its actions on HDL.

When 0.8% POED was added to plasma from healthy subjects, HDL were separated to two peaks with gel filtration column, Protein KW-804 (Showa Denko K.K.). In two HDL peaks, front peak contained a lot of cholesterol, and later peak contained a little. Almost apo A-I and A-II were in late peak. Apo E was also contained in the late peak. POED showed a tendency to bind apolipoprotein-rich HDL.

In the present study, we were unable to identify the HDL to which POED specifically binds. We found, however, that it binds specifically to HDL that contains much apolipoprotein.

Key Words: Apolipoprotein; HDL cholesterol; HDL subclass; HPLC, Polyoxyethylene

1. Introduction

Epidemiological studies have shown that HDL-C is negatively correlated with coronary arteriosclerosis[1),2)]. This is because HDL transports cholesterol from peripheral tissues to the liver, playing a major role in the system for reverse transport of cholesterol (RCT), and having an anti-arteriosclerosis action.

The particle size, lipid content, and apolipoprotein components of HDL is heterogeneous. HDL is separated into large HDL_2 molecules that have low density and small HDL_3 molecules with high density[3),4)]. However, fractions of pre-β-HDL (lipid poor apo A-I)[5),6)] and apolipoprotein E (apo E) rich HDL[7)] have also been reported, and it is not clear which fractions they correspond to. There is also much debate about the clinical significance of measuring HDL subfractions[8-12)].

Density gradient ultracentrifugation is the main method of isolating HDL_2 and HDL_3[13)]. In addition

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to HDL_2 and HDL_3, pre- β -HDL (lipid poor apo A-I) is also isolated using electrophoresis[14]. These methods, however, require special equipment and isolation takes a long time, making them unsuitable for measuring large quantities of samples.

Several kits containing polyoxyethylene derivative (POED) have already been developed to homogeneously measure HDL-C. However, we previously reported that some HDL reacted with POED, and the cholesterol in that HDL is not measured with these kits[15]. Clarification of the characteristics of HDL that is reacted in POED should help to identify problems with HDL-C measurements using POED. Moreover, using those characteristics it may be possible to contribute to the development of a method to measure homogeneous HDL subfractions without the complicated procedures of ultracentrifugation or electrophoresis. In this study we conducted experiments to identify the characteristics of HDL isolated in two peaks in gel filtration columns.

2. Materials and methods

1. Plasma

The plasma used in this study was obtained from healthy adults who had fasted for 12 hours. Written informed consent was obtained from all volunteers. Venous blood was centrifuged at 4℃ and 3,000 rpm for 10 min, and the plasma was collected. One ml of 7.65 mol/L NaBr (density: 1.400) solution was added to 2 ml of plasma and mixed. The plasma with added NaBr was centrifuged (40,000 rpm, 4℃, 24 h), and the majority of plasma protein was removed. The lipoprotein suspended in the supernatant was collected with a Pasteur pipette. The plasma from which the protein had been removed was added to polyoxyethylene tribenzylphenyl ether (Emulgen B66, Kao, Tokyo, Japan), a kind of POED, to a final concentration of 0.8%.

2. Isolating lipoproteins with HPLC

The HPLC method followed the method described by Hara et al.[16, 17]. The HPLC system consisted of two pumps, CCPM-II and CCPE (Tosoh Co. Tokyo, Japan), and UV-VIS DETECTOR/S-3702 (Soma Co., Ltd., Tokyo, Japan). Lipoproteins were separated with two Protein KW-804 columns (Showa Denko K.K., Tokyo, Japan) for gel filtration. The enzymatic reagent for cholesterol measurement was flowed at 0.4 mL/min with CCPE, and the LP-01F (Tosoh Co. Tokyo, Japan) for elution was flowed into columns at a flow rate of 0.7 mL/min with CCPM-II. The enzymatic reagents for total cholesterol assay contained 200 mmol/l phosphate buffer (pH 6.7), detergent (0.1 % Triton X -100, 3 mmol/l sodium cholate), 0.36 mol/l N-ethyl (3-methylphenyl) (N-succinyl) ethylendiamine (EMSE) (Kyowa Medex, Co., Ltd. Tokyo, Japan), 0.5 mmol/l 4-aminoantypirine, 2.5 U/ml peroxidase, 1 U/ml cholesterol oxidase, 0.2 U/ml cholesterol esterase and 1.5 mmol/l NaN_3. The absorbance at 555 nm was continuously monitored after the enzymatic reaction at 37℃ for 5 min in a reactor coil. Lipoprotein isolated by HPLC was collected according to retention time, and the obtained samples were used in electrophoresis and enzyme immunoassay.

3. Electrophoresis and immunoblotting

Samples obtained by HPLC were mixed with electrophoresis sample buffer containing SDS at 1:1. Fifteen μ l of sample was applied to 10% polyacrylamide gel and electrophoresed for 90 min at 20 mA. After electrophoresis was completed, proteins were transferred to the PVDF membrane, and apolipoprotein A-I or A-II antibody were reacted. After reacting with POD-labeled antibody, coloring was done using a Sure Blue Kit (ATTO co. Ltd., Tokyo, Japan).

4. Enzyme immunoassay for apo E-containing HDL

One hundred μ l of goat anti-human apolipoprotein E antibody (Apo Auto Antibody Solution, Sekisui Medical Co. Ltd., Tokyo, Japan: diluted to 1:10000 in carbonate buffer, pH 9.6) was added to Nunc-Immunomodule Maxisorp (Lot 076743: Nalge Nunc International K.K., Tokyo, Japan), and incubated overnight at 4℃. After washing three times with PBS-Tween 20 (0.5% Tween 20 in phosphate buffer saline, pH 7.4), blocking was done using blocking reagent N102 (Hino Ieda Chemicals, Tokyo, Japan). The same plates were directly used after washing.

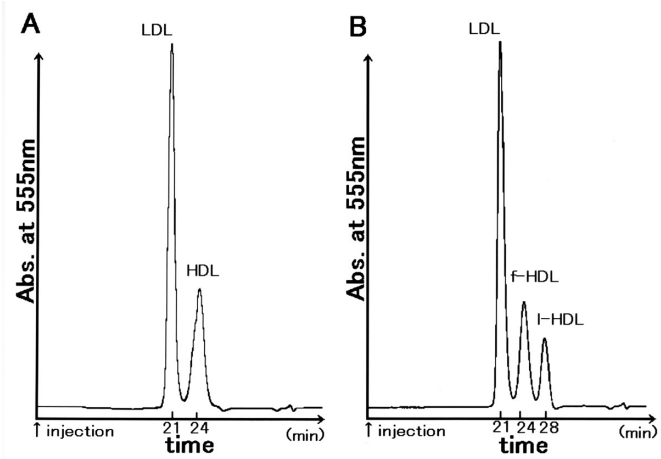

Fig. 1 Changes in HPLC fraction pattern of lipoproteins in plasma with the addition of POED
Two Protein KW-803 (Shodex) columns were used in HPLC as gel filtration columns. TSK-Eluent (Tosoh) was used as the eluent, and eluted at 0.7 ml/min. A total cholesterol coloring solution prepared by the authors was used at 0.4 ml/min. Plasma diluted 10-fold with distilled water (A) and plasma diluted 10-fold with 0.89% polyoxyethylene derivative (POED) (B, final concentration 0.8% POED) were prepared, and the concentrations of cholesterol in each lipoprotein in samples were measured.

Samples collected in HPLC were measured in duplicate. One hundred μl of samples were then added and incubated for 1 h at 37℃. After washing, 100 μl of rabbit anti-human apolipoprotein A-I antibody (Calbiochem: diluted 1:4000 in PBS-BSA) was added to the plates. The solution was then reacted for 1 h at 37℃, after which POD labeled goat anti-rabbit IgG antibody (Calbiochem: diluted 1:10000 in PBS-BSA) was added similarly and the solution was reacted again. After washing, 100 μl of Sure Blue Reserve TMB (Kirkegaard & Perry Laboratories, Inc., Washington DC, US) colorimetric substrate was added, and the reaction was stopped after 30 min with the addition of 100 μl 1 mol/L HCL. The plates were measured at 450 nm, and the concentration of apo E contained in HDL was measured.

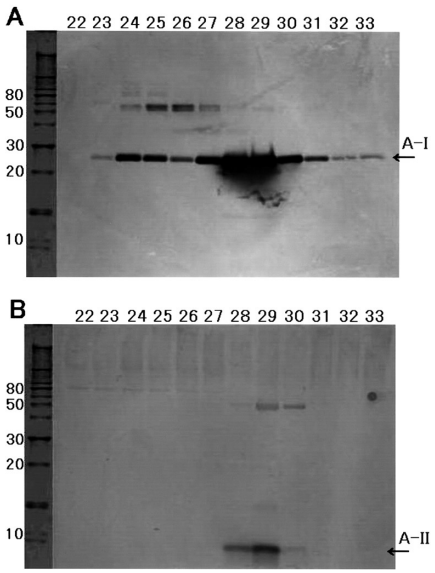

Fig. 2 Distributions of apolipoproteins A-I and A-II in HDL fractions isolated with POED.
Lipoproteins in protein-poor plasma were isolated in gel filtration columns, and fractions were collected every minute starting at 22 min. The HPLC retention time for each lane was recorded. Time of 23-26 min corresponded to f-HDL, and 27-31 min to l-HDL. Immunoblotting was conducted with the use of apo A-I antibodies (A) and apo A-II antibodies (B).

3. Results

1. Changes in HPLC fraction pattern of lipoproteins in plasma with the addition of POED

When lipoprotein was separated with the gel filtration column, LDL appeared first, followed by HDL. When Emulgen B66, a nonionic detergent, was added to plasma, the HDL peak was separated into 2 peaks. There was no change in LDL peak (Fig. 1). HDL

appeared first (f-HDL) was detected in the same retention time (approx. 24 min) as HDL in plasma before the addition of POED. The HDL that appeared next, l-HDL, was seen later (approx. 28 min).

2. Distributions of apolipoproteins A-I, A-II and E in HDL fractions isolated with POED

Plasma was ultracentrifuged to remove proteins that would interfere with the following assays, and protein-poor plasma was obtained. The HDL in protein-poor plasma was also found to be separated into f-HDL and l-HDL, similar to lipoprotein in plasma (data not shown).

HDL in protein-poor plasma was separated into 12 fractions in gel filtration columns. Apolipoprotein A-I (apo A-I) and apolipoprotein A-II (apo A-II) in the 12 fractions obtained were detected with immunoblotting (Fig. 2). Apo A-II was contained only in l-HDL; it was not detected from f-HDL. Apo A-I was included in both HDLs, but more was included in l-HDL.

Next, apo E-rich HDL was measured by enzyme immunoassay (Fig. 3). The peak of apo E-rich HDL had a retention time of 28 min and corresponded to l-HDL peak. Fractions corresponding to f-HDL at a retention time of 23-26 min did not contain much apo E.

4. Discussion

Detergents are often used as a selectively solubilizing agents or inhibitors against enzymatic reaction in methods to homogeneously measure HDL-C or LDL-C[18), 19)]. However, since detergents have a low specificity to lipoproteins, the measurement of other lipoproteins in HDL-C or LDL-C measurement kits is seen as a problem[15)]. We therefore investigated the reactivity to HDL of a detergent, POED (Emulgen B66), that is commonly used to homogeneously measure cholesterol in lipoproteins.

Some of the HDL appeared as a later second peak with the addition of POED. Since it is unlikely that the molecular weight decreased as a result of the action of

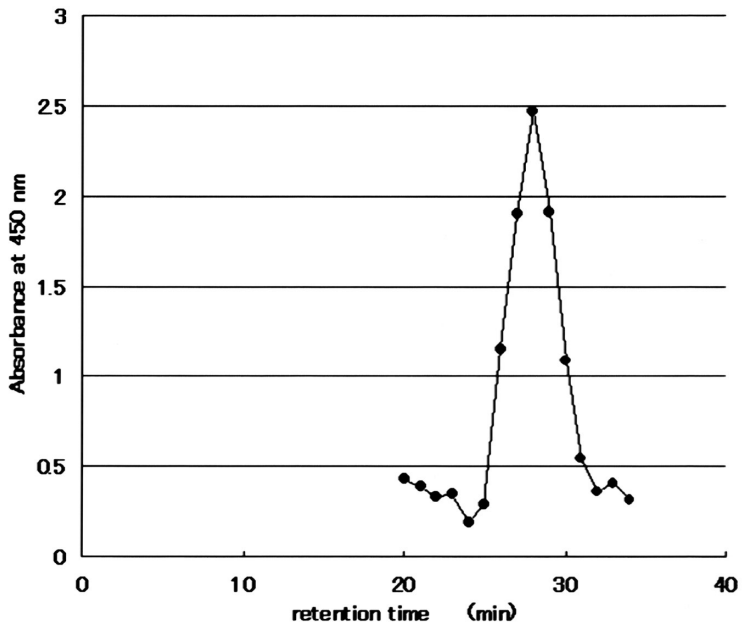

Fig. 3 Distribution of apolipoprotein E in HDL fractions isolated with POED.
Apo E-rich HDL in protein-poor plasma was determined. Apo E-rich HDL was sandwiched between apo A-I antibody and apo E antibody, and POD labeled IgG was reacted. TMB, the substrate of POD, was used for coloring reagent, and the obtained absorbance is shown.

the detergent, it is suggested that there was no change in molecular weight and that the absorption capacity of HDL to the column was changed because of interaction with POED. It has been reported by many researchers that HDL has two main subfractions[3), 4), 20)]. Considering that f-HDL contained much apo A-I and some apo A-II, and that l-HDL contained much FC (data not shown), it is suggested that f-HDL is HDL_2 and l-HDL is HDL_3[21)]. However, according to current reports the prevailing idea is that much apo E is contained in HDL_2[22)], which is the opposite of the present results. Thus, it is difficult to assert that the two kinds of HDL in this study were HDL_2 and HDL_3. Moreover, it is reported that Emulgen B66 solubilizes 95% of HDL_3 and 76% of HDL_2[22)]. Since it is thought that the specificity is low, it may be more effective to measure cholesterol in apo E rich HDL than the fractions of HDL_2 and HDL_3.

It is difficult to homogeneously measure HDL subfractions. These subfractions can be isolated with ultracentrifugation[13)] or capillary electrophoresis[23)], but those procedures take much time and require special equipment and skills. A method to measure cholesterol in HDL_2 and HDL_3 using polyethylene glycol has been developed by Dias et al[24)], but it requires centrifugation[25)]. Moreover, no method has been developed to homogeneously measure cholesterol in apo E rich HDL. The present study may be of some help in developing such a method.

In the present investigation of POED we were unable to identify the HDL to which it specifically binds. We found, however, that it binds specifically to HDL that contains much apolipoprotein. It is known that apolipoprotein determines the function of lipoprotein, and so measurements of apolipoprotein rich HDL using POED should be important. Development of a method to homogeneously measure HDL subfractions by adjusting the POED concentration remains a topic for the future. We concluded that POED bound specifically to HDL that contains much apolipoprotein.

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