Establishment of Monoclonal Antibody Against Human Apo B-48 and Measurement of Apo B-48 in Serum by ELISA Method

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The elevation of chylomicrons and chylomicron remnants in plasma would lead to hyperlipidemia and other complications. Apo B-48, which is translated and produced in the adult intestine from the same gene as Apo B-100, is considered to be an essential component of chylomicrons and chylomicron remnants. Using a peptide representing human Apo B-48 C-terminal sequence as immunogen, we established a monoclonal antibody, B48-151, against human Apo B-48. The specific reactivity for Apo B-48 of this monoclonal antibody was confirmed using Western blot analysis of human plasma in fractions isolated as chylomicron and VLDL. Then, we developed a simple sandwich ELISA method for the detection of human Apo B-48 in serum by combining B48-151 as capturing antibody and HRP-conjugated-polyclonal antibodies for Apo B as signaling antibody. The established sandwich ELISA constitutes a simple method to monitor Apo B-48 level in chylomicrons and chylomicron remnants in human serum. J. Clin. Lab. Anal. 12:289–292, 1998. © 1998 Wiley-Liss, Inc.

Key words: apolipoprotein B-48; chylomicron remnant; postprandial lipidemia; Western blot analysis

INTRODUCTION

Chylomicrons are lipoproteins synthesized exclusively by the intestine to transport dietary fat and fat-soluble vitamins. Among the apolipoproteins found in chylomicrons, such as Apo B-48, A-I, A-IV, and Cs, only the synthesis of Apo B-48 is required for the assembly of chylomicrons (1). Apo B-48 is a 2152 aa long polypeptide translated and produced in the adult intestine from the same gene as Apo B-100 by the action of a series of enzymes (1–3). During circulation in the blood, chylomicrons are exposed to lipolysis and apolipoprotein exchange, and are converted into “chylomicron remnants” (1,4). The removal of chylomicron remnants is a crucial step for human body maintenance because the accumulation of remnants may lead to hypercholesterolemia and may also increase the risk of atherosclerosis. Diabetic lipidemia patients in particular are warned to maintain their remnant level low at postprandial conditions. Therefore, many suggestions and recommendations are documented for controlling the level of cholesterol in plasma chylomicrons and chylomicron remnants (5).

The estimation of the amount of cholesterol in chylomicron remnants is now achieved by removal of nonremnant-like lipoproteins in plasma using a combination of monoclonal antibodies specifically reactive to Apo B-100 and Apo A-I on the solid phase (6). The resulting fraction is then subjected to the measurement of cholesterol level in chylomicron remnants using standard reagents on automated systems. However, the structure of the epitopes on the apolipoproteins and the homogeneity of the epitopes in the plasma lipoprotein population still remain unclear. A more direct assay for Apo B-48 level representing chylomicrons in lipoproteins of plasma is desirable to directly correlate remnant levels.

Another method was developed using polyclonal antibodies against Apo B to measure Apo B-48 level in plasma with a Western blotting technique (7). Other reports describe rabbit polyclonal antibodies selectively reactive to Apo B-48 rather than Apo B-100 (8–10). However, the Western Blotting method is very time-consuming and is less quantitative than the ELISA method. In general, the continuous supply of polyclonal antibodies of equal reactivities for long-term surveys remains questionable. Therefore, in order to confirm the possibility of securing a continuous supply of polyclonal antibodies, we tried to establish monoclonal antibodies against Apo B-48. We utilized a KLH-conjugated Apo B-48 C-terminal peptide as immunogen. Here we report on the first monoclonal antibody against Apo B-48 and its reactivity. We then developed a simple sandwich ELISA method combining this monoclonal antibody to polyclonal antibodies against Apo B to measure Apo B-48 in human serum.

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MATERIALS AND METHODS

Blood Samples

Ten healthy volunteers aged 24–36 (five males and five females) were enrolled for quantitation of Apo B-48 level with their informed consent. Blood was drawn at fasting stage and after 1.5 hr and 4 hr of meal (not controlled) supply, and their serum was then stored in a frozen state for Apo B-48 measurement.

Peptides

C-LQTYMI (C6), C-QTYMI (C5), C-TYMI (C4), and LQTYMIQFDQYI were synthesized on a peptide synthesizer (Shimadzu, Model PSSM-8, Kyoto, Japan) with a standard program, purified with HPLC on a 5C18-AR 300 column (Nacalai Tesque, Kyoto, Japan) and conjugated with KLH by the action of GMBS in DMF/0.1 M phosphate buffer (pH 7.5).

Preparation of Lipoproteins From Human Plasma

We utilized the method of R.J. Havel et al. (11) for the preparation of lipoproteins, with minor modifications. Ultracentrifugation using the Beckman Model X-80 at 26,000 $\times g$ for 1 hr gave a chylomicron fraction in the form of an upper and lower layer which were further centrifuged with d = 1.006 solution at 11,400 $\times g$ for 20 hr, giving rise to VLDL fraction. Then, further centrifugation with d = 1.063 at 114,000 $\times g$ for 20 hr gave an LDL fraction.

Purification of Apo B-48

A chylomicron fraction was collected from human plasma and separated electrophoretically on SDS-PAGE. Then, the gel site representing Apo B-48 according to the molecular marker was sliced and eluted to collect the desired Apo B-48 as standard. Apo B-100 was purchased from Chemicon (Temecula, CA) as standard.

Monoclonal Antibody Preparation

Each peptide, C4-KLH, C5-KLH, and C6-KLH, was emulsified with Freund's complete adjuvant and immunized into BALB/c mouse. After a boosting with each immunogen, fusion of spleen cells with myeloma cells (P3U1) was carried out with PEG (1500, Behringer, Germany) followed by screening for desired hybridomas reactive only to Apo B-48. The Western blotting method was utilized for screening. Thus, chylomicron and VLDL fractions were separated on SDS-PAGE (3–15% gradient gel) and electrophoretically blotted onto a nitrocellulose membrane. Immunoreaction was carried out with rabbit antimouse Ig-HRP conjugate (DAKO Japan, Kyoto, Japan) as a secondary antibody with 4-Chloronaphtol as substrate.

The inoculation of the established hybridoma cells into mice to obtain the ascites followed by the purification with protein A-Sepharose column gave the desired monoclonal antibody, B48-151.

The subclass of monoclonal antibody B48-151 was also determined by an isotyping kit (Amersham, Buckinghamshire, UK) and its IgG_{2b},κ was found. The inhibition assay with peptides (5 µg/ml) was also carried out on the nitrocellulose membrane for Western Blot.

Monoclonal and Polyclonal Antibodies Against Apo B

In order to compare the reactivity of B48-151 against Apo B-100/Apo B or Apo B-48, monoclonal (B014) and polyclonal antibodies (goat) against human Apo B were purchased from Chemicon International Inc HRP-conjugated Apo B polyclonal antibodies were purchased from Binding Site (Birmingham, UK).

RESULTS

The addition of a cysteine moiety to the amino terminal position of each peptide representing Apo B-48 C-terminal for conjugation was designed to create a free carboxy terminal end. The respective immunization of C5-KLH and C6-KLH resulted in antibodies of almost equal reactivities by Western blotting assay. However, C4-KLH did not result in antibodies reactive to Apo B-48 strongly enough to show a clear band by western blot analysis. We chose cell lines derived from C6-KLH immunogen for further screening of monoclonal antibodies reactive to Apo B-48.

The specificity of monoclonal antibody B48-151 was characterized as follows: chylomicron (CM), VLDL, and LDL fractions were loaded into SDS-PAGE (3–15% gradient gel) and blotted onto a nitrocellulose membrane. Postcoating of a membrane with skim milk (1%) followed by immunoreaction with MAb B48-151 and rabbit antimouse IgG-HRP conjugate as a secondary antibody gave a clear band, as shown in Figure 1. To compare the reactivity of MAbs, we utilized MAb B014 (Chemicon), which recognizes Apo B-100 and goat polyclonal antibodies against human Apo B (Chemicon) as our control.

This MAb B48-151 clearly demonstrated its reactivity against components within VLDL and chylomicron fractions. Furthermore, the immunoreaction of MAb B48-151 for Apo B-48 on a membrane was confirmed by the inhibitory effect of the free peptide C6 by Western blot analysis, as shown in Figure 2.

The inhibition of the Western blotting assay of Apo B-48 was observed by the sole addition of C6 peptide, which has a free C-terminal portion. However, the other peptides, LQTYMIQFDQYI (overlapping peptide of C-term of Apo B-48 and a continuous part of B-100) (12) did not show any inhibitory activity (data not shown).

We then tried to develop a simple sandwich ELISA using

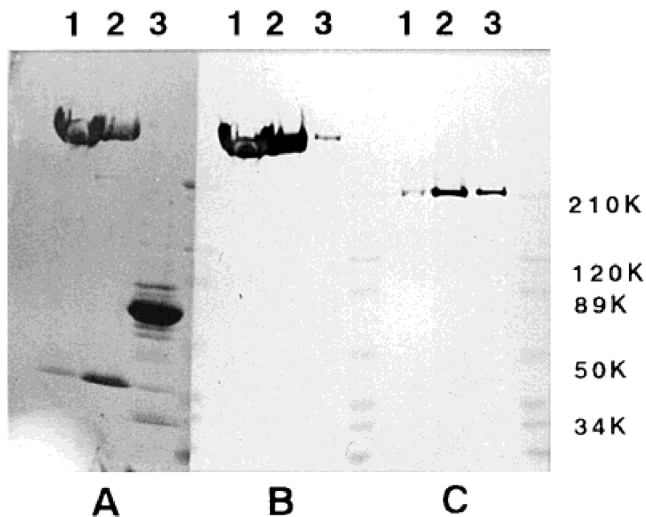

Fig. 1. Western blot analysis of LDL, VLDL, and CM fractions with anti-Apo B-100 and MAb, B48-151. Lanes 1, 2, and 3 represent the fraction of LDL, VLDL, and CM, respectively. Panels **A, B,** and **C** were the result of CBB staining pattern, the anti-Apo B-100 staining pattern, and B-48-151 staining pattern. The molecular markers were also shown.

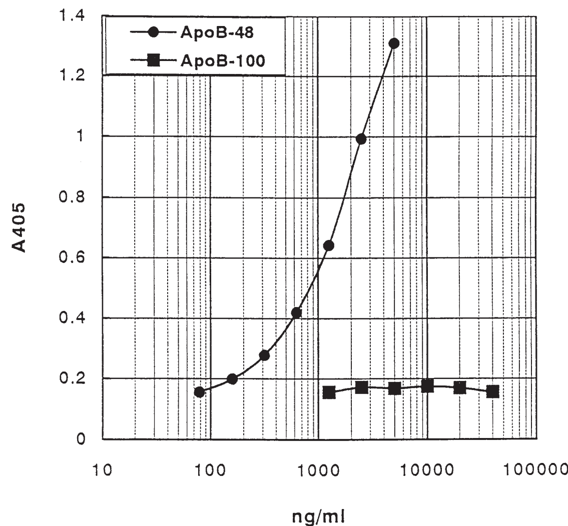

Fig. 3. Standard curve of Apo B-48 and Apo B-100 on the established ELISA using B48-151 monoclonal antibody as capturing antibody. ● represents Apo B-48 and ■ represents Apo B-100.

B48-151 as capturing antibody on a 96-well plate and HRP-conjugated polyclonal antibodies for Apo B. We utilized purified Apo B-48 from human plasma as standard. We obtained a standard curve for Apo B-48 but could not detect any Apo B-100 on this ELISA system as shown in Figure 3.

Therefore, the established ELISA was confirmed as a simple method specific for the direct measurement of Apo B-48.

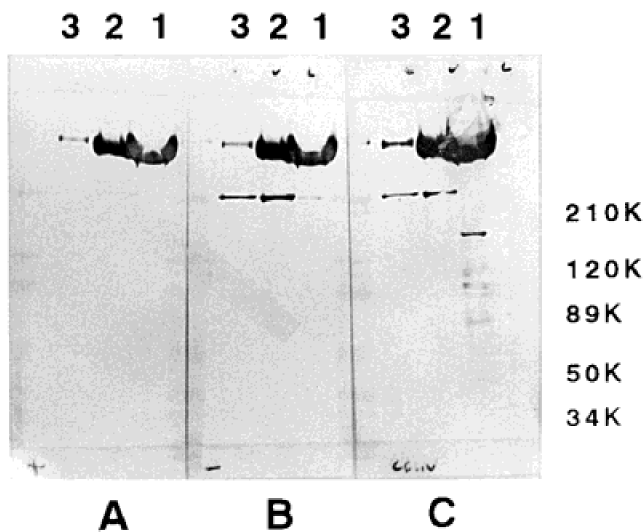

Fig. 2. Immunoreactivities of anti-Apo B on Western blotting pattern of Apo B-48 on the membrane in the presence of C6-peptide. Lanes 1, 2, and 3 represent LDL, VLDL, and CM, respectively. Panels **A, B,** and **C** represent the immunoreaction pattern: A, monoclonal antibodies B48-151 + B014 in the presence of C6-peptide (5 μg/ml; B, B48-151 + B014 without peptide; and C, goat anti-Apo B.

We then extended the application of this ELISA method to the monitoring of the postprandial state in ten healthy individuals. Thus, we compared the Apo B-48 level between fasting and postprandial pair sera. The results are shown in Figure 4. Results clearly indicate that the Apo B-48 level in serum was increased in postprandial state for each individual.

DISCUSSION

Emergent needs for the clinical monitoring of cholesterol level to avoid risk factors of atherosclerosis were documented. Recently, a method for the measurement of the cholesterol level in chylomicron remnants was developed using a subtraction method of nonremnant-like particles from plasma lipoprotein particles by a combination of monoclonal antibodies

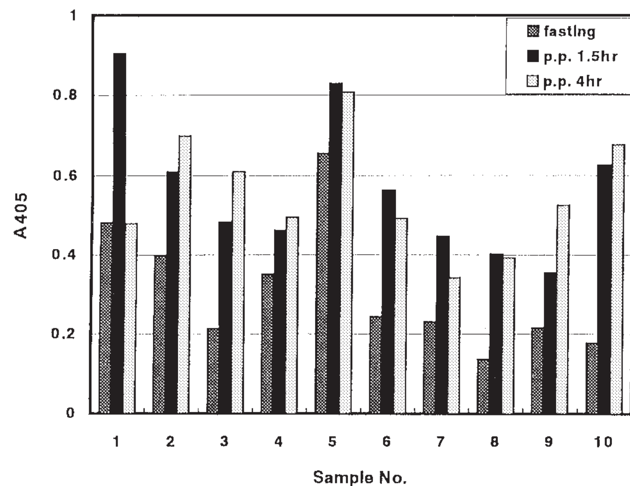

Fig. 4. Histogram of the Apo B-48 level between fasting stage and postprandial stage of each individual.

to capture Apo B-100 and Apo A-I on the solid phase (6). However, this method was discarded due to the critical assay conditions needed for capture and because the heterogeneity of the epitope structures of apolipoproteins in lipoproteins remains unclear.

Another approach based on the Western blot technique was also developed using antibodies against Apo B with an enhanced chemiluminescent detection method on membranes (7). Originally, polyclonal antibodies reactive to both Apo B-100 and Apo B-48 were used. However, their reactivity to Apo B-48 was dependent upon the preparation conditions of the sample and the separation of Apo B-100 and Apo B-48 on the gel using this technique. Several groups reported polyclonal antibodies reactive to Apo B-48 rather than Apo B-100 (8–10). However, there is no guarantee that such polyclonal antibodies of equal reactivities may be easily reproduced for long-term surveys. Furthermore, the Western blot technique is a rather complicated method and somewhat less quantitative than the ELISA method. Therefore, a more simple and quantitative way of measuring the level of Apo B-48 in human serum is being sought.

We estabished a B48-151 monoclonal antibody and a simple ELISA method for the detection of human Apo B-48 in serum requiring no extraction steps. We utilized the same immunogen as that of Peel's publication (8). As described above, we established the desired monoclonal antibody against human Apo B-48 C-terminal portion.

As shown in Figures 1 and 2, the MAb B48-151 was reactive against VLDL fractions, which is quite surprising because it was reported previously that VLDL does not possess any Apo B-48. Here, we simply separated the fractions showing differences in particle size and weight. Our VLDL fractions may have been contaminated with chylomicron remnants to show immunoreactivity against Apo B-48. Furthermore, their lower reactivity against chylomicron appears to be due to the low concentration of chylomicrons in the sample specimen.

As shown in Figure 3, we could detect Apo B-48 in the range of 150 ng/ml–5 μg/ml here, but we could not detect any amount of Apo B-100 with this ELISA method. As mentioned above, the overlapping peptides, including the successive 6 aa long peptide after the C-terminal position of Apo B-48, stretching a total of 12 aa, did not show any reactivity against this established monoclonal antibody, B48-151. Therefore, the plausible epitope was mapped in a region which includes the free carboxy terminal end.

There is an urgent need to control the level of cholesterol in plasma remnants to avoid risk factors leading to atherosclerosis. We have not yet optimized the assay conditions of this ELISA. However, the results obtained with the pair samples of healthy volunteers suggested that this method may be a suitable and simple way to monitor the Apo B-48 level of patients. We compared the cholesterol level in the remnant-like particles of each sample to confirm the high correlation between two different types of analyses (detailed data will be published elsewhere). Although the optimization of ELISA conditions may be required for further discussion in clinical applications, this simple ELISA technique may offer a very useful method for monitoring diabetic meritus patients. We are currently in the process of doing a complete study on subjects, establish reference ranges and study the lipid profile in addition to the measurement of Apo B-48. The results of this study will be reported elsewhere.

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