Isolation and Characterization of Apolipoprotein B48-Containing Lipoproteins with a Monoclonal Antibody Against Apolipoprotein B48

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Aim: Remnant lipoproteins are well known to play a pivotal role in atherosclerosis. In patients with postprandial dyslipidemia, metabolic pathways for exogenous lipoproteins are generally disturbed, resulting in accumulation of chylomicron remnants. Although it has been difficult to make a specific antibody against apolipoprotein B48 (apoB48), a constituent of exogenous lipoproteins, we succeeded in creating a specific monoclonal antibody against apoB48. In this study, we isolated apoB48-containing lipoproteins from lipoproteins with a density less than 1.019 g/mL using this anti-apoB48 monoclonal antibody (4C8).

Methods: Apolipoproteins and lipids were analyzed to confirm whether apoB48-containing lipoproteins are isolated from other lipoproteins. The characteristics of apoB48-containing lipoproteins in the plasma of patients were compared with type 2 diabetes mellitus and non-diabetic patients. Furthermore, the uptake of apoB48-containing lipoproteins by THP-1 cells (a human acute monocytic leukemia cell line), HepG2 cells (a human hepatoma cell line), and human umbilical vein endothelial cells (HUVEC) was investigated. Also, the expression of apoB48 receptors in these cells was tested with RT-PCR.

Results: Apolipoprotein analysis of 4C8-bound lipoproteins indicated the isolation of apoB48-containing lipoproteins, because the content of apoB100 was quite low (less than 5%). Compared with lipoproteins that were not bound to the antibody, apoB48-containing lipoproteins had a higher content of triglycerides. There was no significant difference in the composition of apoB48-containing lipoproteins between patients with and without type 2 diabetes. Uptake of fluorescence-labeled apoB48-containing lipoproteins by THP-1-derived macrophages and HepG2 cells, but not by HUVEC, was observed. The specificity of this uptake was confirmed because the fluorescent signal was competed out by an excess amount of the same unlabeled lipoproteins. RT-PCR revealed the expression of apoB48 receptors in THP-1 and HepG2 cells but not in HUVEC. These results suggest that specific uptake of apoB48-containing lipoproteins may occur via apoB48 receptors.

Conclusion: ApoB48-containing lipoproteins have a higher triglyceride content and are taken up into THP-1-derived macrophages and HepG2 cells by a specific pathway.


Key words: Apolipoprotein B48, Chylomicron remnant, Apolipoprotein B48 receptor

Introduction

In recent years, attention has been focused on remnant lipoproteins as atherogenic lipoproteins in patients with dyslipidemia\textsuperscript{1}. There are two types of remnants, chylomicron remnants (CMR: exogenous lipoproteins derived from the small intestine) and very low density lipoprotein (VLDL: endogenous lipoproteins derived from the liver) remnants. In patients with postprandial hyperlipidemia, the metabolic pathways for exogenous lipoproteins are mainly disturbed, resulting in the accumulation of CMR, which may be
related to persistent hypertriglyceridemia and atherosclerosis\(^2\). It used to be necessary to perform complicated examinations, such as a fat-loading test, to make a diagnosis of postprandial hyperlipidemia\(^3,4\). Currently, remnant-like lipoproteins (RLP), which are the fraction that is not bound to 2 monoclonal antibodies of anti-apolipoprotein A1 (apoA1) and anti-apolipoprotein B100 (apoB100), are used clinically as a marker of postprandial hyperlipidemia\(^5\). It has been reported that the fasting RLP concentration is an independent risk factor for coronary artery disease\(^6,7\); however, since RLP contain various lipoproteins, including VLDL remnants, measurement of RLP does not strictly reflect exogenous lipoproteins, such as chylomicrons (CM) and CMR. It is known that apolipoprotein B48 (apoB48), which is a specific protein of CM and CMR, accounts for 48% of the molecular weight of apoB100 and that the two apolipoproteins have the same N-terminal amino acid sequence\(^8\). Because of their similarity, it has been difficult to raise an antibody that recognizes apoB48 alone; however, we succeeded in preparing an apoB48-specific monoclonal antibody\(^9\). Because it is now possible to measure the plasma apoB48 concentration by ELISA using this monoclonal antibody, apoB48 could be used as a marker of postprandial hyperlipidemia. In this study, we isolated apoB48-containing lipoproteins using our anti-apoB48 monoclonal antibody (4C8) and investigated their characteristics. Furthermore, the uptake of apoB48-containing lipoproteins by THP-1 cells (a human acute monocytic leukemia cell line), HepG2 cells (a human hepatoma cell line), and human umbilical vein endothelial cells (HUVEC) was tested, and the expression of apoB48 receptors\(^10\) in these cells was examined by RT-PCR.

**Materials and Methods**

**Materials**

Coupling of 4C8 to gel was performed using CNBr Activated-Sepharose 4 Fast Flow Lab Packs (GE Healthcare Bioscience Co., Ltd., Tokyo, Japan). HUVEC, HepG2 cells, and THP-1 cells were purchased from the Health Science Research Resources Bank (Japan Health Sciences Foundation, Tokyo, Japan). Human vascular endothelial cell serum-free basic culture medium, basic fibroblast growth factor (bFGF), recombinant human epidermal growth factor (EGF), human plasma fibronectin, and RPMI 1640 medium were purchased from Invitrogen (CA, USA). Dulbecco’s modified Eagle’s medium was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from JRH Biosciences (KS, USA). Penicillin and streptomycin, which were added to culture medium throughout this study, were obtained from Invitrogen. Lipoprotein-deficient serum (LPDS) was prepared from FBS by ultracentrifugation as described previously\(^11\). Total cholesterol (TC), TG, and phospholipids (PL) were measured using assay kits from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipoproteins were fluorescence-labeled with an Alexa Fluor 488 Protein Labeling Kit (Invitrogen). RNA was extracted using TRIzol (Invitrogen). To perform RT-PCR, RNasin Plus RNase Inhibitor and M-MLV Reverse Transcriptase RNase H Minus were obtained from Promega (WI, USA), while Oligo Pd(N)\(^6\) was purchased from Amersham (Uppsala, Sweden). KOD DNA polymerase was from Toyobo Co., Ltd. (Osaka, Japan). Primers for RT-PCR to detect apoB48 receptor mRNA were obtained from Sigma Genosys Japan (Tokyo, Japan).

**Isolation of ApoB48-Containing Lipoproteins**

Blood was collected from patients with type 2 diabetes mellitus and non-diabetic patients who were attending the outpatient clinic of our hospital after overnight fasting. Lipoprotein fractions with a density (d) ≤ 1.019 g/mL were separated by ultracentrifugation from pooled serum samples of these patient groups\(^12\). After dialysis against PBS, the lipoproteins were incubated with 4C8 conjugated gel for 24 hours at 4°C and then centrifuged at 3,000 rpm for 2 min at 4°C. Lipoproteins in the supernatant were employed as 4C8-nonbound lipoproteins. After the gel was washed 3 times with PBS, lipoproteins bound to 4C8 gel were uncoupled with 5 volumes of 3 M sodium thiocyanate. After dialysis against PBS, the dialysate was concentrated by ultrafiltration and then used as 4C8-bound lipoproteins.

**Composition of ApoB48-Containing Lipoproteins**

To confirm the apoprotein composition of the isolated proteins, polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 5–22.5% gradient gel, followed by staining with Coomassie brilliant blue. TC, TG, and phospholipids (PL) in the d ≤ 1.019 g/mL lipoprotein fractions, 4C8-nonbound lipoproteins, and 4C8-bound lipoproteins were then measured with assay kits (Wako Pure Chemical Industries, Ltd.). The protein content was determined by the method of Bradford\(^13\) using bovine serum albumin (BSA) as the standard.

**Uptake of ApoB48-Containing Lipoproteins by THP-1 Cells, HepG2 Cells, and HUVEC**

The protein moieties of apoB48-containing lipo-
proteins were labeled using an Alex Fluor 488 Protein Labeling Kit and uptake experiments were performed. Low-density lipoprotein (LDL; density of 1.019–1.063 g/mL) fractions were collected from the above-mentioned pooled serum samples by ultracentrifugation. THP-1 cells were seeded into 24-well plates (3.2 × 10^5 cells/well) with 10% FBS-supplemented RPMI 1640 medium and transformed into macrophages (MΦ) by incubation for 48 hours with 100 nM phorbol 12-myristate 13-acetate (PMA). HepG2 cells were seeded into 24-well plates (3.2 × 10^5 cells/well) and incubated for 24 hours with 10% FBS-supplemented Dulbecco’s modified Eagle’s medium. HUVEC were seeded into 24-well plates (3.2 × 10^5 cells/well) and incubated for 48 hours with human vascular endothelial cell medium containing 20 mg/mL bFGF, 10 ng/mL EGF, 10 μg/mL fibronectin, and 10% FBS. Each type of cell was washed twice with PBS, and then incubated for 4 hours in medium containing 50 mg/mL LPDS. To investigate the uptake of apoB48-containing lipoproteins, the cultured cells were divided into the following 4 groups: (1) negative control group (PBS and the buffer used for fluorescence labeling were added at the same volumes as in other groups), (2) fluorescence-labeled apoB48-containing lipoprotein (11.4 μg/mL as protein) group, (3) fluorescence-labeled apoB48-containing lipoprotein (11.4 μg/mL as protein) + unlabeled apoB48-containing lipoprotein (57 μg/mL, i.e., a molar amount 5-fold higher than that of fluorescence-labeled apoB48-containing lipoproteins) group, and (4) fluorescence-labeled apoB48-containing lipoprotein (11.4 /mlμg as protein) + LDL (114.0 μg/mL, i.e., a molar amount 5-fold higher than that of fluorescence-labeled apoB48-containing lipoproteins) group. After these lipoproteins were added to the cultures for 10 min, the cells were washed 3 times with PBS, and fluorescent signals were detected by fluorescence microscopy (LX70; Olympus Corporation, Tokyo, Japan).

Investigation of ApoB48 Receptor Expression

Cells (MΦ-transformed THP-1 cells, HepG2 cells, and HUVEC) were seeded into 6-well plates at a density of 1.5 × 10^6/well. After incubation for 24 hours in 10% FBS-supplemented medium, it was changed to medium containing 50 mg/mL LPDS, in which cells were cultured for another 24 hours. Total RNA was extracted using TRIzol reagent. The concentration of RNA was measured by spectrophotometry, and 5 μg RNA was subjected to reverse transcription using RNasin Plus RNase Inhibitor, M-MLV Reverse Transcriptase RNase H Minus, and Oligo PD(N), to synthesize cDNA. To detect apoB48 receptor mRNA, PCR was performed using KOD DNA polymerase and the following primers: 5prime;-ACAGACAGGATGGACTTCCT-3 (sense) and 5prime; TTCCCAGCTTCCTCAGCCTCT-3prime; (antisense). PCR products were run on 2% agarose gel, which was stained with ethidium bromide.

Results

Isolation and Composition of ApoB48-Containing Lipoproteins

SDS containing polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 4C8-bound lipoproteins, 4C8-nonbound lipoproteins, and lipoproteins with d ≤ 1.019 g/mL from patients with type 2 diabetes and nondiabetic patients. As shown in Fig. 1, apoB100 was the main constituent of 4C8-nonbound lipoproteins, and apoB48 was almost undetectable. On the other hand, 4C8-bound lipoproteins were composed of apoB48 (approximately 48%) and apoE (approximately 48%), while apoB100 was almost undetectable. Densitometry indicated that apoB100 accounted for 4.2% of 4C8-bound lipoproteins in patients with type 2 diabetes, while it was undetectable in nondiabetic patients. Based on these results, 4C8-bound lipoproteins were used as apoB48-containing lipoproteins in subsequent experiments.

Protein/Lipid Composition of ApoB48-Containing Lipoproteins

Table 1 shows the protein/lipid composition of each type of lipoprotein. ApoB48-containing lipoproteins had a TG content of 89.8%, while that of 4C8-
nonbound lipoproteins was 64%. Thus, the TG content of apoB48-containing lipoproteins was clearly higher. Comparison of apoB48-containing lipoproteins between patients with type 2 diabetes and non-diabetic patients showed a high TG content in both groups and there was no significant difference in lipoprotein composition between groups.

### Table 1. Protein/lipid composition (%w/w) of 4C8-nonbound lipoproteins and apoB48-containing lipoproteins (TG: triglycerides, TC: total cholesterol, PL: phospholipids)

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>TG</th>
<th>TC</th>
<th>PL</th>
</tr>
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<tr>
<td><strong>Non-diabetes mellitus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonbound lipoproteins</td>
<td>10.0</td>
<td>64.0</td>
<td>11.0</td>
<td>15.0</td>
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<tr>
<td>ApoB48-containing lipoproteins</td>
<td>3.8</td>
<td>89.8</td>
<td>2.5</td>
<td>4.0</td>
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<tr>
<td><strong>Diabetes mellitus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonbound lipoproteins</td>
<td>7.5</td>
<td>56.3</td>
<td>12.0</td>
<td>24.2</td>
</tr>
<tr>
<td>ApoB48-containing lipoproteins</td>
<td>3.9</td>
<td>88.5</td>
<td>2.4</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Uptake of ApoB48-Containing Lipoproteins by MΦ-Transformed THP-1 Cells, HepG2 Cells, and HUVEC

**Fig. 2** shows the uptake of fluorescence-labeled apoB48-containing lipoproteins by MΦ-transformed THP-1 cells. As shown in Photo B, signals were observed in the cells after fluorescence-labeled apoB48 lipoproteins were added to cultures. As shown in Photo C, no signals were observed when unlabeled apoB48-containing lipoproteins were added simultaneously at a 5-fold higher molar excess. As shown in Photo D, a decrease of intracellular fluorescence was not noted when LDL was added along (at a 5-fold molar excess) with fluorescence-labeled apoB48-containing lipoproteins.

**Fig. 3** shows the uptake of apoB48-containing lipoproteins by MΦ-transformed THP-1 cells.

MΦ-transformed THP-1 cells were incubated with PBS and buffer used for fluorescence labeling (A: negative control), fluorescence-labeled apoB48-containing lipoprotein (11.4 μg/mL) (B), fluorescence-labeled apoB48-containing lipoprotein (11.4 μg/mL) + unlabeled apoB48-containing lipoprotein (57 μg/mL) (C), and fluorescence-labeled apoB48-containing lipoprotein (11.4 μg/mL) + LDL (114.0 μg/mL) (D). After these lipoproteins were added to the cultures for 10 min, the cells were washed 3 times with PBS, and fluorescent signals were detected by fluorescence microscopy.
lipoproteins by HepG2 cells. Specific uptake of these lipoproteins was observed in HepG2 cells as well as MΦ-transformed THP-1 cells. Uptake of apoB48-containing lipoproteins by HUVEC was not detected (Fig. 4).

**ApoB48 Receptor Expression**

To clarify the mechanism involved in the uptake of apoB48-containing lipoproteins by cells, the expression of apoB48 receptors was investigated by RT-PCR. The expected product was 539 bp in size. As shown in Fig. 5, apoB48 receptor expression was detected in HepG2 cells and MΦ-transformed THP-1 cells. Expression of apoB48 receptor was not detected in HUVEC (data not shown).

**Discussion**

Many reports have been published recently since the hypothesis was advanced that an increase of TG-rich apoB-containing lipoproteins was involved in the onset of atherosclerosis. Epidemiological studies have shown a correlation between hypertriglyceridemia and coronary artery disease. Increased amounts of oxidized LDL and small dense LDL are found in patients with hypertriglyceridemia with an increment of remnant lipoproteins, and these are all known as atherogenic lipoproteins. In particular, CMR are considered to play a very important role in the onset of atherosclerosis. We succeeded in preparing a monoclonal antibody that specifically recognized apoB48. A synthetic peptide corresponding to the 10 amino acids of apoB48 C-terminal was used as the antigen for this monoclonal antibody. Uchida et al. also created a monoclonal antibody using the 8 C-terminal amino acids by the similar method, and ELISA to measure apoB48 was also reported. Our anti-apoB48 monoclonal antibody (4C8) thus obtained does not recognize apoB100 despite its similarity to apoB48. The antibody, 4C8, recognizes apoB48, but not apoB100, presumably because the three-dimensional structure of the C-terminus of apoB48 (the epitope) is structurally masked in apoB100. When lipoproteins were retrieved using this monoclonal antibody, apoB48 was prominent and apoE was also detected among apolipoproteins; therefore, isolation of apoB48-containing lipoproteins was confirmed. Because 4C8 recognizes the three-dimensional structure of the apoB48 C-terminus, it can be suggested that this C-terminus protrudes from the surface of the lipoprotein.
Lipoproteins containing apoB48 are mostly CM and CMR. In general, TG accounts for at least 80% of CM, along with cholesterol esters, free cholesterol, phospholipids, and proteins, so CM are rich in TG. The main structural apolipoprotein is apoB48, while apoA1, AII, CII, CIII, and E are also found. The TG

Fig. 4. Uptake of apoB48-containing lipoproteins by HUVEC.
HUVEC were incubated with PBS or lipoproteins using the same procedure as in Fig. 2.

Fig. 5. Expression of apoB48 receptors of HepG2 cells and Mφ-transformed THP-1 cells.
PCR was performed to detect apoB48 receptor mRNA. The expected PCR product was 539 bps in size.
content was at least 80% for the apoB48-containing lipoproteins isolated in the present study, which was higher than in IDL or VLDL. This indicated that the isolated lipoproteins were very similar to CM.

Diabetes mellitus is a typical disease associated with lipid abnormalities, such as hypertriglyceridemia and low HDL-cholesterol, as well as an increase of remnant lipoproteins. It was shown by the JDCS and a Japanese study, that the incidence of coronary artery disease is higher in patients with type 2 diabetes. This may be partly due to the high levels of remnants (apoB48-containing lipoproteins) or to the presence of qualitatively different remnants in patients with type 2 diabetes. It has been reported that apoB48 lipoproteins are significantly increased in patients with type 2 diabetes compared with healthy people; however, this study found no appreciable difference in the composition of apoB48-containing lipoproteins between patients with type 2 diabetes and nondiabetic patients. This suggests that elevated remnant lipoproteins accompanied by hypertriglyceridemia in type 2 diabetes represent an increase in the number of remnant lipoprotein particles rather than an increase in the TG content of remnant lipoproteins.

MΦ-transformed THP-1 cells and HepG2 cells showed the specific uptake of fluorescence-labeled apoB48-containing lipoproteins. When an excess of LDL was simultaneously added, the uptake of fluorescence-labeled apoB48-containing lipoproteins showed no change, suggesting that the uptake of apoB48-containing lipoproteins occurs via a different route from that for the uptake of LDL. It is thought CMR bind to LDL receptors and LDL receptor-related proteins (LRP) via apoE as a ligand. The apoB48-containing lipoproteins isolated in the present study were shown to be apoE-rich by SDS-PAGE. In recent years, the existence of a receptor for TG-rich lipoproteins on macrophages with apoB48 as a ligand has been reported and this is known as the apoB48 receptor. In this study, expression of the apoB48 receptor in MΦ-transformed THP-1 cells and in HepG2 cells was shown, but HUVEC did not express this receptor. Brown et al. reported the expression of apoB48 receptors by MΦ-transformed THP-1 by HUVEC, but not by HepG2 cells. This different result was presumably ascribable to differences in the conditions of cell culture and the PCR primers used, but it will be necessary to perform further studies to identify the reasons. Since the uptake of apoB48-containing lipoproteins and expression of the apoB48 receptor in MΦ-transformed THP-1 cells was detected in both studies, it seems that exogenous lipoproteins can induce atherosclerosis (including transformation of macrophages to foam cells) in patients with postprandial hyperlipidemia. As described above, apoB48-containing lipoproteins contains apo E, so it is also possible that they were taken up to MΦ-transformed THP-1 cells and HepG2 cells by an apo E-dependent pathway. The mechanism of the uptake of apoB48-containing lipoproteins to these cells should be determined in the future.

Recently, we detected apoB48 in human atherosclerotic plaques using 4C8, the antibody employed in this study; therefore, the role of apoB48-containing lipoproteins in atherosclerosis may be found to be substantial.

The apoB48-containing lipoproteins isolated in the present study were obtained from pooled serum samples of patients with type 2 diabetes or nondiabetic patients, being purified at an extremely low yield from a large volume of pooled serum. Since it was impossible to investigate apoB48-containing lipoproteins in individual patients, data on the lipid composition and other factors could not be subjected to statistical analysis; however, similar results were obtained in duplicate experiments (data not shown). In the future, it will be necessary to improve the yield and clarify how apoB48-containing lipoproteins differ between diseases, as well as defining their role in the onset of atherosclerosis.

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