The Lipoprotein Fraction between VLDL and LDL Detected by Biphasic Agarose Gel Electrophoresis Reflects Serum Remnant Lipoprotein and Lp(a) Concentrations

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We analyzed lipoprotein profiles in 616 Japanese by biphasic agarose gel electrophoresis using Chol/Trig Combo™ to yield HDL, VLDL, LDL and CM fractions which were stained with cholesterol and triglyceride reagents, respectively. To further evaluate the pattern of electrophoresis, we analyzed the fraction between VLDL and LDL to confirm the possibility of a MidBand by using an automatic-five-fraction function. The cholesterol concentrations in MidBand (MidBand-C) showed a good correlation to remnant-like particle-cholesterol (RLP-C) ($r = 0.95$) in 23 consecutive samples (TC < 220 mg/dl, Lp(a) < 30 mg/dl). However, MidBand-C concentrations of subjects with high Lp(a) levels (Lp(a) > 30 mg/dl) were also high compared to RLP-C concentrations. The average MidBand-C levels in elderly normolipidemic control subjects (TC < 220, TG < 150) were 5.2 ± 2.4 mg/dl in 30 males (mean age, 70 ± 10 years) and 5.4 ± 2.0 mg/dl in 40 females (64 ± 11 years). The average MidBand-C levels of normolipidemic patients with coronary artery diseases (CAD; TC < 220, TG < 150) were 9.4 ± 4.1 mg/dl in 126 males (mean age, 66 ± 10 years) and 9.1 ± 4.0 mg/dl in 44 females (67 ± 10 years). These levels were significantly higher than control values ($p < 0.0001$). Areas under ROC curves were greater for MidBand-C than for TC, LDL-C and TG when used to discriminate between the patients with CAD and normolipidemic control subjects for each sex. These results suggest that the MidBand-C level may be useful as an indicator of risk for CAD. *J Atheroscler Thromb*, 2006; 13: 55–61.

Key words: Agarose gel electrophoresis, Automatic five-fraction, Remnant like particles-cholesterol, Coronary artery disease.

Introduction

The major remnant lipoproteins are a mixture of the exogenous chylomicron remnant derived from the intestine and the endogenous very low density lipoprotein (VLDL) remnant (IDL) derived from the liver. Methods have been developed to measure remnant lipoproteins. Remnant lipoproteins have been identified as atherogenic (1).

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We reported that serum levels of remnant-like particle cholesterol (RLP-C) were increased in patients with diabetes and impaired glucose tolerance (2). We also reported that remnant lipoproteins had unique biological effects such as the induction of apoptosis of cultured endothelial cells (3). VLDL remnants can be separated by the ultracentrifugal method at a density of 1.006–1.019 g/ml (4). However, this method is not suitable for routine analysis, because it takes hours, and the processing of multiple specimens is difficult (5).

Agarose gel electrophoresis is an authentic method of detecting remnant lipoprotein which appears as a broad band. The mid-band in polyacrylamide gel electrophoresis is also well known as the band of remnant lipoprotein (4). However, these methods are qualitative not quantitative. Recently a quantitative assay for RLP-C has been developed (6), and an elevation of plasma RLP-C levels was reported in patients with coronary artery diseases (CAD) (7). But there are considerable numbers of patients with type I and V hyperlipidemia whose levels of RLP-C appear abnormal in immunoadsorption assays with antibody. Therefore, it is important to develop simple and comprehensive methods to measure remnant lipoprotein levels.

We analyzed the lipoprotein fractions by using the agarose gel electrophoresis with Chol/Trig Combo™, which can quantify each lipoprotein fraction on the basis of the separate staining of cholesterol and TG and by automatic analysis. Based on this method, it is possible not only to measure the levels of HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) quantitatively, but also to recognize the entire pattern of lipoprotein fractions (8–11). We identified the fraction between the VLDL and LDL fractions using the Automatic five-fraction function of Chol/Trig Combo™ and elucidated that this fraction was closely related to the levels of RLP-C and increased in CAD patients compared with normolipidemic subjects. We named this fraction MidBand and clarified its clinical significance.

Methods

Study subjects

We measured RLP-C and Lp(a) concentrations in 64 patients [31 males (mean age 60 ± 11 years, age range 31–81 years) and 33 females (63 ± 16 , 26–80 years)], who requested a RLP-C test between 2000.03 and 2003.03.

The elderly control subjects were the 80 normolipidemic (TC 114–219 mg/dl, TG < 150 mg/dl) staff members of the Kobe University hospital enrolled in an annual medical checkup and patients 60 years or older who were free from CAD, liver disease, kidney disease and diabetes mellitus who were selected from among patients at Kobe University Hospital. These were 35 males (mean age 66 ± 13 years, age range 41–90 years) and 45 fe-

males (64 ± 12 , 50–88 years)

To evaluate the clinical usefulness of MidBand-C in CAD, 168 patients with CAD (TC 80–219 mg/dl, TG < 150 mg/dl) were admitted to Kobe University hospital and their diagnosis was confirmed by coronary angiography. This group consisted of 125 males (mean age 67 ± 10 years, age range 35–88 years) and 43 females (68 ± 9 , 47–83 years).

Hospital staff were ordered with informed consent to come to blood sampling in the fasting state. Sera from the patients with CAD were drawn in a 12-hour fasted state with venopuncture. These samples were made anonymous without linking.

Biochemical analysis

Serum total cholesterol (TC) and TG concentrations were measured using an auto analyzer TBA-80M (TOSHIBA, Tokyo, Japan) by the enzymatic method. The RLP-C concentration was measured by the immune adsorption method (JIMROII, Japan). The serum Lp(a) concentration was measured using an auto analyzer TBA-80FR (TOSHIBA) by the latex agglutination immunoassay.

Agarose gel electrophoresis analysis

The serum samples were subjected to the lipoprotein analysis using agarose gel electrophoresis (Rapid Electrophoresis, Helena Laboratories, Beaumont, Texas) and the gels were stained with cholesterol and TG reagent. The conditions for electrophoresis were as follows; serum application volume was 1 μ l, and electrophoresis time was 18 min at 400 volts, and 20°C. Elution profiles were analyzed by an automatic densitometer, Chol/Trig Combo™ (Helena Kennkyusyo, Saitama, Japan).

Apolipoprotein B48 (apoB48) and Apolipoprotein B100 (B100) were identified with immunoblotting on the nitrocellulose film where the agarose gels were transferred. For detecting apo B48 and apo B100, we used anti-apoB48-151 monoclonal antibody (Fujirebio Co, Tokyo, Japan) and anti-apo B100 monoclonal antibody (Biochemical Division, Ohio), respectively. Ofuto® cream (Milk fat 35%, 547 kcal. Jyomou Shokuhin company, Gunma, Japan) was used in the fat load. The samples which could not be measured on the day were stored at –40°C with the addition 1/20 (vol/vol) of dimethyl sulfoxide (8). This preservation method was effective up to four weeks (data not shown).

Statistical analysis

Data are expressed as the mean $\pm$ SD. Pearson's Correlation coefficient was used for the relationship between MidBand-C and RLP-C. Differences were examined with Student's *t* test. The computer analysis was done using Stat View Ver. 5.0. A *p*-value less than 0.01 was considered statistically significant. Receiver-operative charac-

Fig. 1. Electrophoregram of cholesterol fractions, triglyceride fractions, apo B48 or apo B100. Apo B48 and apo B100 were detected by immunoblotting with anti -apo B48 or anti-apo B100 antibody. Sera were electrophoresed 4 hours after fat load (Ofuto™)

Fig. 2. The representative densitometric scanning patterns for Chol/Trig Combo™. (A): cholesterol stain (B): triglyceride stain (C): layer in A and B (D): an example of a normolipidemic control subject, TC 202, TG 86, HDL-C 65, Mid Band-C 2.0, RLP-C < 2.5 (mg/dl)

teristics curve analysis was used to show performance figures. For the computation and analysis of ROC curves, we used the software program Med Calc, Ver. 6.01 (Med Calc Software).

Results

Determination of MidBand

We established automatic-five-fraction on the strength of the electric charge with apolipoprotein. The fraction eluted between the VLDL and LDL fractions in agarose gel electrophoresis of Chol/Trig Combo™ was defined as MidBand.

To confirm whether MidBand was rich in apoB48, serum was selected 4 hours after the loading of fat (Ofuto™) and electrophoresed and transferred to a nitrocellulose membrane. Nitrocellulose membrane was immunoblotted with anti-human apoB48 antibody and compared with the electrophoregram of cholesterol fractions, TG fractions and fractions immunoblotted with anti apoB100 antibody. MidBand was rich in apoB48, and the LDL fraction was rich in apoB100 (Fig. 1).

For example, agarose gels were stained for cholesterol (Fig. 2A) or TG (Fig. 2B) after electrophoresis and analyzed by the automatic densitometer Chol/Trig Combo™. After both patterns were imposed as shown in Fig. 2C,

Fig. 3. Yellow dots: A significant positive correlation between MidBand-C levels and RLP-C levels when TC < 220 mg/dl and Lp(a) < 30 mg/dl.

Red dots: Lp > 30 mg/dl

Blue dots: TC > 220 mg/dl and Lp(a) < 30 mg/dl

RLP-C: Remnant-like particle cholesterol

MidBand was determined as the fraction between LDL and VLDL fractions. A representative profile of a normolipidemic subject is shown in Fig. 2D. The MidBand-cholesterol (MidBand-C) concentration was calculated from the serum cholesterol level and percentage of MidBand in the cholesterol fraction of the agarose gel electrophoretogram.

Relationship among MidBand-C, RLP-C and Lp(a)

To evaluate the relationship between MidBand-C and RLP-C, we measured RLP-C concentrations of 64 subjects and compared them to MidBand-C concentrations. We selected 23 subjects with both a TC of less than 220 mg/dl and Lp(a) concentration of less than 30 mg/dl out of the 64 subjects. As shown in Fig. 3 (yellow dot), there was a significant positive correlation between concentrations of MidBand-C and RLP-C in subjects with a correlation coefficient (r) of 0.95 ($p < 0.001$). However, MidBand-C concentrations of hyperlipidemic patients deviated from the standard line (Fig. 3, red dot and blue dot). MidBand-C concentrations of subjects with high Lp(a) levels were also high compared with RLP-C concentrations. As we have already reported, Lp(a) at levels of more than 30 mg/dl migrates as a peak of cholesterol between VLDL and LDL using this method (13). This position is the same as that of MidBand. Representative elution profiles are shown in Fig. 4. In sample A, the Lp(a) concentration was 142 mg/dl showing a sharp MidBand-C peak. Sample B was from a type IIb hyperlipidemic patient and MidBand-C is the mid-band. Sample C was obtained from a type III hyperlipidemic patient and had

a broad beta-band in the lipoprotein fraction. Sample D showed the presence of chylomicron and a high concentration of VLDL-TG, indicating type V hyperlipidemia.

Comparison of MidBand-C levels of normolipidemic subjects with patients with CAD

Table 1 shows a summary of the clinical characteristics of the elderly control subjects and the patients with CAD. Significant differences in HDL-C and MidBand-C concentrations were observed between the two groups in each sex.

To elucidate the clinical significance of MidBand-C in CAD, we measured the MidBand-C concentrations of the patients with CAD. Mean MidBand-C concentrations were 9.4 ± 4.1 and 9.2 ± 4.0 mg/dl in the male and female patients, and significantly higher than in control subjects ($p > 0.0001$) (Fig. 5).

A ROC analysis was performed for MidBand-C, HDL-C, LDL-C, TC and TG in the CAD patients and control subjects in males (Fig. 6) and females (Fig. 7). The area under the ROC curve (AUC) for MidBand-C in males and females was 0.80 and 0.79, respectively, showing a significant difference from that for LDL-C, TC and TG. ROC curves of HDL-C and MidBand-C almost overlapped and there was no significant difference.

Discussion

In the present study, we demonstrated that MidBand-C concentrations analyzed by agarose gel electrophoresis and automatic densitometer showed a significant correlation with RLP-C concentrations. MidBand is consid-

Fig. 4. Densitometric scanning patterns of hyperlipidemia.

Patient A had type IIa hyperlipidemia, and the mid-band; TC 311, TG 41, RLP-C 2.9, MidBand-C 39.5, Lp(a) 142 mg/dl. Patient B had type IIb hyperlipidemia, and the mid-band; TC 252, TG 264, RLP-C 9.0, MidBand-C 46.4 mg/dl. Patient C had type III hyperlipidemia, and a broad beta-band in the lipoprotein fraction; TC 232, TG 342, RLP-C 12.0, MidBand-C 58.0 mg/dl. Patients D had the type V hyperlipidemia, and an increase of chylomicron; TC 167, TG 967, RLP-C 71.0, MidBand-C 14.0 mg/dl.

Table 1. Clinical characteristics of CAD patients and elderly control subjects

	Men		Female	
	control (n = 35)	CAD (n = 125)	control (n = 45)	CAD (n = 43)
Age (year)	66.1 ± 12.6	66.7 ± 9.6	63.7 ± 11.5	68.1 ± 9.3
TC (mg/dl)	164.7 ± 30.7	169.3 ± 29.7	178.7 ± 22.3	173.3 ± 24.5
TG (mg/dl)	86.0 ± 28.8	92.2 ± 30.7	92.0 ± 23.4	98.5 ± 28.3
LDL-C (mg/dl)	104.7 ± 24.2	103.9 ± 26.4	92.1 ± 20.8	104.5 ± 22.5
HDL-C (mg/dl)	61.3 ± 14.4	42.4 ± 13.3*	69.4 ± 17.5	47.4 ± 13.9**
MidBand-C (mg/dl)	5.2 ± 2.3	9.4 ± 4.1*	5.5 ± 2.0	9.2 ± 3.9**

* Significantly different from the value of control men, $p < 0.0001$

** Significantly different from the value of control female, $p < 0.0001$

ered similar to the mid-band observed in polyacrylamide gel electrophoresis (PAGE). However, it is not possible to conduct a quantitative analysis of remnant lipoproteins by PAGE. Additionally, preparative and analytical ultracentrifugation methods have been used to separate VLDL and IDL. However these methods are labor-intensive and complex. With our method using Chol/Trig Combo™, the operation is simple and semi-automated. Moreover, one is able not only to recognize the entire lipoprotein profile but also to quantitate the cholesterol and TG of MidBand that is equivalent to the IDL or remnant lipoprotein. We demonstrated that the MidBand

richly contained apo B48.

To investigate the relation between MidBand-C and RLP-C, we measured RLP-C concentrations of 23 subjects with both a TC concentration of less than 220 mg/dl and a Lp(a) concentration of less than 30 mg/dl. In these subjects, there was a significant correlation between RLP-C and MidBand-C. Moreover, Lp(a) at levels of more than 30 mg/dl migrates as the same peak as MidBand-C. These results suggested that MidBand-C reflected VLDL remnants and Lp(a). Both are known to be atherogenic.

We analyzed MidBand-C in 80 elderly control subjects. To investigate the significance of the MidBand-C con-

Fig. 5. Levels of MidBand-C in the elderly normolipidemic control subjects and the normolipidemic patients with CAD. There was a significant difference in MidBand-C levels between the control and CAD in each sex.

Fig. 6. and Fig. 7. Receiver operating characteristic curves of MidBand-C, HDL-C, LDL-C, TC and TG when used to discriminate between the patients with CAD and control subjects in males and females. AUC: Area under the curve.

centration in CAD, we measured levels in 168 patients with CAD. Mean MidBand-C levels of CAD patients (9.4 ± 4.1 mg/dl and 9.2 ± 3.9 mg/dl in males and females, respectively) were significantly higher than those of elderly control subjects (5.2 ± 2.3 mg/dl and 5.5 ± 2.0 mg/dl, $p < 0.0001$). In the ROC analysis performed on CAD patients and control subjects, the AUC for MidBand-C was 0.80 for males and 0.79 for females. In addition, the AUC of MidBand-C was significantly higher than that for LDL-C, TC or TG, suggesting that the elevation in the concentration of MidBand-C could be a useful marker of the risk of developing CAD.

Considering these results, measurements of MidBand-C made using Chol/Trig Combo™ may be important to evaluate the risk of CAD.

Conclusion

We defined MidBand as the fraction between LDL and the VLDL fractions in Chol/Trig Combo™. MidBand-C reflected the serum levels of RLP-C and Lp(a), and may be an important index with which to estimate the risk of CAD.

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