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Circadian change of serum concentration of small dense LDL-cholesterol in type 2 diabetic patients

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ABSTRACT

Background: Type 2 diabetic patients have a higher risk of atherosclerosis than non-diabetic subjects. This difference may be attributable to increased levels of small dense low-density lipoprotein-cholesterol (sLDL-C) in diabetic patients. As the sLDL-C concentration is elevated in hypertriglyceridemia, which is exaggerated postprandially, we examined whether the sLDL-C level increases postprandially in type 2 diabetes.
Methods: We obtained 7 blood samples (30 min before and 2 h after each meal, and at midnight) from 15 patients with diabetes and ten normal controls. Following the precipitation of very low-density lipoprotein and large buoyant LDL (bLDL) with heparin–Mg^{2+}, the sLDL-C concentration was determined as the cholesterol concentration by a homogeneous assay.
Results: The fasting sLDL-C concentration was 60.3% higher in the diabetic patients than in the controls (1.01 ± 0.21 vs. 0.63 ± 0.21 mmol/l, $p<0.001$). The sLDL-C concentrations in both groups were highest in the fasting state, decreased after breakfast, and remained low until midnight. The maximal reduction in the absolute sLDL-C concentration was 56.5% greater in the diabetic patients than in the controls (0.36 ± 0.13 vs. 0.23 ± 0.16 mmol/l, $p<0.05$). Thus, the sLDL-C/bLDL-cholesterol (bLDL-C) ratio was reduced with increases in bLDL-C.
Conclusions: The sLDL-C concentration decreases postprandially in diabetes. This absolute reduction in sLDL-C may contribute to an acceleration of atherosclerosis in diabetic patients.

1. Introduction

Cardiovascular disease remains the leading cause of death in Japanese as well as Caucasian patients with type 2 diabetes. Although blood glucose control significantly reduces microvascular complications, it is not sufficient to reduce macrovascular complications [1]. Moreover, diabetic patients have a higher risk of atherosclerosis than non-diabetic subjects with the same low-density lipoprotein (LDL)-cholesterol levels [2]. These differences may be related to increased remnant lipoprotein levels [3], decreased high-density lipoprotein-cholesterol (HDL-C) levels [4], or persistent chronic subclinical inflammation [5]. Another possible factor is the high concentration of small dense LDL-cholesterol (sLDL-C) in diabetic patients [6]. Previous *in vitro* experiments have shown that small dense LDL (sLDL) is more susceptible to oxidative stress than large buoyant LDL (bLDL) [7]. Once sLDL is oxidized, it is recognized by scavenger receptors and taken up by macrophages into atherosclerotic lesions [8].

Accumulating evidence indicates that an increased sLDL-C level is associated with hypertriglyceridemia, which is the most common form of dyslipidemia in diabetes [9]. Consistent with the high incidence of hypertriglyceridemia in this group, the sLDL-C concentration is also higher in diabetic patients than in non-diabetic subjects. In the postprandial state, hypertriglyceridemia is more exaggerated in diabetic patients than in non-diabetic subjects. However, there are currently no reports of postprandial changes in the sLDL-C concentration in diabetes. The objective of this study was to examine whether the sLDL-C level increases during the daytime in diabetic patients. We measured the sLDL-C concentrations seven times daily in diabetic patients as well as control subjects by a combination of the precipitation method and homogeneous assay. Our data clearly indicate that type 2 diabetic patients have a greater reduction in sLDL-C than control subjects in the postprandial state.

Abbreviations: LDL-C, low-density lipoprotein-cholesterol; sLDL-C, small dense LDL-cholesterol; bLDL-C, buoyant LDL-cholesterol; VLDL, very low-density lipoprotein; TGRL, triglyceride-rich lipoprotein; LPL, lipoprotein lipase; HL, hepatic lipase; CETP, cholesteryl ester transfer protein.

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2. Materials and methods

2.1. Study subjects and blood sampling

The study subjects comprised 2 groups: type 2 diabetic patients (DM group; 9 men and 6 women) and normolipidemic healthy subjects (control group; 8 men and 2 women). To confirm the accuracy of the method used to measure sLDL-C by comparison with ultracentrifugation, we obtained blood samples from outpatients with various disorders (outpatient group; 7 men and 6 women). The patients in the DM group were treated with oral hypoglycemic agents ($n=4$; sulfonylurea 1, glinides 2, thiazolidine + α-glucosidase inhibitor 1), insulin ($n=5$), or changes in diet ($n=6$). Some of the subjects were also treated with a statin [$n=3$; pravastatin (5 mg) 2, atorvastatin (10 mg) 1], or fibrate ($n=1$; bezafibrate 200 mg). To elucidate the circadian rhythm of sLDL-C, seven blood samples were drawn per day at 30 min before and 2 h after each meal (07:30, 10:00, 11:30, 14:00, 17:30, and 20:00 h) and at midnight, and the sLDL-C concentrations were determined as described below. For most of the laboratory tests, including sLDL-C, the samples were left at room temperature until completely coagulated to avoid contamination with fibrin. Next, the serum was separated by centrifugation at 3000×*g* for 10 min and stored at 4 °C until assayed. For the measurement of glycosylated hemoglobin (HbA1c) and blood glucose (BG), the samples were placed in glass tubes containing EDTA-K_2 and NaF to inhibit glucose catabolism. The patients in the DM group ate only meals provided by the hospital kitchen and stayed at the hospital during the study. Their daily calorie intake was set at 25.0–27.5 kcal/kg/day (lipids 38–54 g, proteins 59–77 g, and carbohydrates 150–250 g). The healthy subjects ate regular meals three times per day, corresponding to an intake of approximately 2000 kcal/day (lipids 54 g, proteins 82 g, and carbohydrates 290 g), and were allowed to engage in low-level activities. Informed consent was obtained from all subjects. The study protocol was approved by the ethics committee of Niigata City General Hospital and complied with the Declaration of Helsinki.

2.2. Determination of the sLDL-C and large buoyant LDL-cholesterol (bLDL-C) concentrations

The sLDL-C concentrations in the samples were determined using the precipitation/homogeneous assay (P/H) method described by Hirano et al. [10]. This method consists of 2 steps. First, very low-density lipoprotein (VLDL) and large bLDL were precipitated with heparin–Mg^{2+} and removed by centrifugation at 15,000×*g* for 15 min at 4 °C (microcentrifuge MRX-150, Tomy Disital Biology, Tokyo, Japan). The cholesterol concentration in the supernatant was measured by the homogeneous assay for LDL-cholesterol (sd LDL-C, Denka Seiken, Tokyo, Japan). The inter- and intra-assay CVs for the P/H method were 4.0 (mean sLDL-C = 1.45 mmol/l) to 4.5% (mean sLDL-C = 0.819 mmol/l) and 1.3 (mean sLDL-C = 1.86 mmol/l) to 2.9% (mean sLDL-C = 0.69 mmol/l), respectively. For determining the bLDL-C concentration, total LDL-cholesterol (LDL-C) was measured by the homogeneous assay (LDL-Ex, Denka-Seiken). The bLDL-C concentration was calculated by subtracting sLDL-C from the total LDL-C.

In the outpatient group, we measured the dense LDL subclass by 2 different methods for comparison. First, the LDL subclass with a density range of 1.044 to 1.063 was isolated by sequential ultracentrifugation [11]. The top fraction (LDL-C_{ultra}) was carefully recovered, and the cholesterol concentration was measured by the enzymatic method. The sLDL-C concentration was also determined by the P/H method using the same blood samples.

2.3. Other laboratory measurements

Using a Hitachi-7450 automated analyzer, we measured the total cholesterol (TC) and triglyceride (TG) concentrations by enzymatic methods, the high-density lipoprotein-cholesterol (HDL-C) concentration by the homogeneous assay (HDL-Ex, Denka Seiken), the BG concentration by the hexokinase method, and the albumin concentration by the bromocresol green method (Clinimate ALB, Sekisui Medical, Tokyo, Japan). The apolipoprotein (Apo)AI, ApoB, and ApoE concentrations were determined using a commercial turbidimetric immunoassay kit (Sekisui Medical). The HbA_1c concentration was determined by high-performance liquid chromatography. The inter- and intra-assay CVs were <1% for TC, TG, HDL-C, BG, albumin, ApoAI, ApoB, ApoE, and HbA_1c. For some of the study subjects, the cholesteryl ester transfer protein (CETP) mass was measured by enzymatic immunoassay (CETP-ELISA Daiichi, Sekisui Medical). The inter- and intra-assay CVs for the CETP mass were 5.5% and 2.9%, respectively.

2.4. Correcting the lipoprotein concentration

When the study subjects were awake during the day, they moved freely throughout the hospital. Therefore, we used the albumin concentration to correct for the effects of postural changes on the lipoprotein concentration. The corrected values were obtained by the following formula: $[\text{Lipoprotein}]_{\text{corrected}} = [\text{Lipoprotein}]_{\text{point i}} \times [\text{Albumin}]_0 \div [\text{Albumin}]_{\text{point i}}$, where $[\text{Lipoprotein}]_{\text{point i}}$ is the lipoprotein concentration at point i, $[\text{Albumin}]_{\text{point i}}$ is the albumin concentration at point i, and $[\text{Albumin}]_0$ is the albumin concentration at baseline [12].

2.5. Statistical analysis

Statistical analyses were performed using Microsoft Excel 2007 (Microsoft Japan, Tokyo, Japan) with the Statcel add-in software for Excel (OMS, Tokorozawa, Japan). The data are presented as the means ± SD unless otherwise stated. To assess the correlation between sLDL-C and LDL-C_{ultra}, Pearson's single linear regression analysis was performed. To ensure the use of a suitable analytic method, we examined whether the data showed a Gaussian distribution. Paired *t*-tests were used to compare the concentrations of TG, TC, HDL-C, CETP, and LDL subfractions in the same subjects, while Student's or Welch's *t*-tests were used to compare the lipoprotein concentrations among the different groups. For all analyses, a $p<0.05$ was considered significant.

3. Results

3.1. Lipoprotein profiles of the study subjects

The DM group had significantly higher fasting BG, HbA_1c, LDL-C, ApoB, and TG concentrations compared with the control group (Table 1). The HDL-C and ApoAI concentrations in the DM group were lower than those in the control group. The combination of hypertriglyceridemia and hypo-α-lipoproteinemia is typical among patients with diabetes.

3.2. Correlation between sLDL-C and LDL-C_{ultra}

In the outpatient group ($n=13$), the sLDL-C concentration as determined by the P/H method showed a good correlation with the LDL-C_{ultra} as determined by ultracentrifugation ($r=0.92$, $p<0.001$).

3.3. Fasting sLDL-C and bLDL-C concentrations

In the fasting state, the distributions of LDL particle size differed significantly between the DM and control groups. The sLDL-C concentration was 60.3% higher in the DM group than in the control group (1.01 ± 0.21 vs. 0.63 ± 0.21 mmol/l, $p<0.001$), while there was no significant difference in bLDL-C between the two groups (Table 1). Thus, the sLDL-C/bLDL-C ratio in the DM group was 47.4% higher than that in the control group (Table 1).