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Metformin reduces circulating malondialdehyde-modified low-density lipoprotein in type 2 diabetes mellitus

Abstract

Purpose: Type 2 diabetes is known to be associated with increasing cardiovascular mortality. Malondialdehyde-modified LDL (MDA-LDL) is an oxidized LDL and is increased in patients with diabetes or hypertriglyceridemia. Elevated MDA-LDL has been reported to be a risk factor of atherosclerosis or cardiovascular disease. Sitagliptin is a dipeptidyl peptidase-4 inhibitor and a new class of hypoglycemic agents. In this study, the effects of increasing the dose of metformin and add-on sitagliptin on MDA-LDL were examined in type 2 diabetes patients.

Methods: Seventy patients with type 2 diabetes, inadequately controlled despite on-going treatment with metformin 500 mg/day, were enrolled in this randomized controlled trial. The patients received additional metformin (500 mg/day) or sitagliptin (50 mg/day) for 6 months, and changes in metabolic parameters including MDA-LDL were evaluated.

Results: After 6 months of treatment, add-on sitagliptin (n=35) improved fasting blood glucose (FBG) and hemoglobin A1c (HbA1c) to significantly greater extent than increasing the dose of metformin (n=35). There were no differences in total cholesterol and low-density lipoprotein cholesterol levels between two groups. MDA-LDL levels (mean±S.E.) decreased significantly with increasing the dose of metformin (from 94.40±6.35 to 77.83±4.74 U/L, P<0.005), but remained unchanged with add-on sitagliptin treatment (from 89.94±5.59 to 98.46±6.78 U/L, p>0.05). Multiple linear regression analysis identified increasing the dose of metformin treatment as the only independent factor associated with decreased MDA-LDL (β coefficient 0.367, P<0.0119), and no significant correlation between change in MDA-LDL and fasting blood glucose or HbA1c.

Conclusion: These results suggest that increasing the dose of metformin improves serum MDA-LDL levels in type 2 diabetes mellitus.

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Cardiovascular disease is the leading cause of death among type 2 diabetic patients [1]. Intensive glycemic control reduces microvascular complications, but the effect on macrovascular changes and cardiovascular disease is rather limited [2]. Controlling glucose levels represents one of the principal treatment goals of diabetes therapy, but the favorable effects of improved glycemic control on macrovascular complications of diabetes has been difficult to generalize [3, 4]. The United Kingdom Prospective Diabetes Study demonstrated that the risks of cardiovascular morbidity and mortality were reduced in patients with type 2 diabetes mellitus receiving intensive glucose control with metformin [5]. Metformin improves insulin sensitivity and is important in the management of traditional cardiovascular risk factors such as high hemoglobin (Hb)A1c level, dyslipidemia, hypertension and central obesity, all of which are associated with insulin resistance [6].

Some non-traditional cardiovascular risk factors have been reported. Oxidized low-density lipoprotein (LDL), which is one type of modified LDL, is considered to play a key role in the progression of atherosclerosis [7]. Malondialdehyde-modified LDL (MDA-LDL) is an oxidized LDL and is increased in patients with diabetes or hypertriglyceridemia [8]. Elevated MDA-LDL has been reported to be a risk factor of atherosclerosis or cardiovascular disease [9]. Furthermore, circulating MDA-LDL levels correlate negatively with peak sizes of LDL particles [10]. Metformin enlarges LDL particle size in type 2 diabetes patients [11]; however, the effect of metformin on serum MDA-LDL levels has not been reported.

Sitagliptin is a highly selective dipeptidyl peptidase-4 (DPP-4) inhibitor and has been available in Japan since 2009. Dipeptidyl peptidase-4 is an enzyme involved in the degradation of the incretin hormones, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1), from active hormones to inactive metabolites. GIP and GLP-1 are released into circulation by the intestine in response to a meal, and both hormones increase glucose-dependent insulin secretion. Sitagliptin increases active incretin concentration to inhibit the degradation of active incretins, thereby enhancing their glucoregulatory effects [12-14]. Sitagliptin, both as monotherapy and combination therapy with other antihyperglycemic agents, has been shown to improve glycemic control and β cell function and to be well-tolerated in type 2 diabetes patients in large multinational, placebo-controlled and active-controlled trials [15, 16]. Furthermore, sitagliptin has also been reported to improve lipid profile and reduce oxidative stress [17]. Thus, sitagliptin is expected to reduce the risk factors of cardiovascular disease including MDA-LDL; however, the effect of sitagliptin on MDA-LDL has not been fully discussed.

In the present study, we hypothesized that increasing the dose of metformin or adding sitagliptin to on-going metformin therapy reduces MDA-LDL. Type 2 diabetic patients were treated with increasing the dose of metformin or add-on sitagliptin and the effect of these therapies on serum MDA-LDL levels evaluated.

Methods

Subjects

The protocol of the study was prepared in accordance with the Helsinki Declaration and was approved by the institutional review board at Sakura Hospital, Toho University Medical Center (No. 2012-112). Informed consent was obtained from all subjects: before participation, the purpose of this study was explained to each subject, and consent was obtained for both participation in the study and for release of the study data.

Seventy patients with type 2 diabetes that was inadequately controlled despite on-going treatment with metformin 500 mg/day, and who attended Sakura Hospital, Toho University Medical Center, were enrolled. The patients were divided into two randomized groups using the envelope method. Both groups continued to take metformin 500 mg/day, and the two groups were given different additional therapies as follows: increasing the metformin dose from 500 to 1000 mg/day in one group (increasing the dose of metformin group, $n = 35$), and adding sitagliptin 50 mg/day on top of metformin 500 mg/day in the other group (add-on sitagliptin group, $n = 35$). Both the subjects and investigators were not blinded to the treatment. A statement of American Diabetes Association (ADA) and the Europe Association for the Study of Diabetes (EASD) recommend that the first line of hypoglycemic agent is metformin, so patients who were already being treated with metformin were included in the study [18]. Table 1 shows the clinical characteristics of the subjects at baseline.

The subjects were observed for 6 months after initiation of increasing the dose of metformin or add-on sitagliptin therapy. The following parameters were measured before and after 6 months in this study: body weight, body mass index (BMI), fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), immunoreactive insulin (IRI), homeostasis model assessment-insulin resistance (HOMA-IR), serum total cholesterol level (TC), serum triglycerides level (TG), serum high-density lipoprotein-cholesterol level (HDL-C), serum low density lipoprotein-cholesterol level (LDL-C) and serum MDA-LDL level. During the study period, all patients maintained their same diet and exercise therapies, and did not change medications including statins. All subjects received nu-

tritional guidance from a dietitian every month. The dietitian analyzed the meals of the patient recorded in a diary and suggested changes if necessary.

Body weight measurement and blood sampling

Body weight was measured and blood samples were collected in the morning after 12 hours of fasting. Serum was separated within 1 hour, and the sample was used for measurements of HbA1c and serum lipids.

Measurement of HbA1c and plasma lipid concentrations

For HbA1c measurement, blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). The stable and unstable fractions of HbA1c were measured by high-pressure liquid chromatography method using the Hi-Auto A1c kit (Kyoto Daiichi Kagaku, Kyoto, Japan). Data of the stable form were used in the present analysis. The value of HbA1c was expressed according to the National Glycohemoglobin Standardization Program (NGSP) [19]. HOMA-IR was estimated from FBG and IRI.

Plasma TC and TG levels were measured enzymatically using kits from Nippon Shoji Co., Ltd. (Osaka, Japan) and a Hitachi 7150 analyzer (Hitachi, Ltd., Tokyo, Japan). HDL-C was measured using a selective inhibition assay (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan) [20]. Serum LDL-C levels were calculated using the Friedewald formula.

Measurement of malondialdehyde-modified LDL

The ELISA used for MDA-LDL measurement was based on the method reported by Kotani et al. [21]. In brief, micro-titer plates were coated with a monoclonal antibody against MDA-LDL. Duplicate 100 μ L samples were added to the wells and incubated for 1 h at room temperature. After washing, a β -galactosidase-conjugated monoclonal antibody against apo lipoprotein B (apo B) was added to each well, and the mixture was incubated for 1 h at room temperature. After washing, 100 mL of the substrate solution, o-nitrophenyl- β -galactopyranoside (10 mmol/L), was dispensed into each well and allowed to react for 2 h at room temperature. The reaction was terminated by adding a stop solution, and absorbance was determined spectrophotometrically at 415 nm. The primary standard used was artificially prepared MDA-LDL in which 15% of the amino groups were modified. From a calibration curve constructed using this standard, the amounts of MDA-LDL in the samples were determined. One unit per liter was defined as the absorbance obtained with the standard at a concentration of 1 mg/L.

Outcome measures

The primary outcome measures were serum MDA-LDL levels and changes in MDA-LDL levels in the two groups after 6 months of intervention compared to baseline. Secondary outcomes were indicators of glucose and lipid metabolism and other clinical parameters.

Statistical analysis

Data are expressed as mean $\pm$ SD, unless indicated otherwise. Normal distribution was tested by Shapiro-Wilk test. Some data were not normally distributed. Normality was secured by a logarithmic transformation. Statistical analysis was performed using Student's t-test. Multiple linear regression analysis was performed using the JMP computer software version 9.0 (SAS, Cary, NC, USA). P values less than 0.05 were considered significant.

Results

Baseline characteristics in increasing the dose of metformin group and add-on sitagliptin group

With the exception of HDL-C levels, clinical and biochemical parameters at baseline were not significantly different between the two groups. HDL-C was significantly higher in the add-on sitagliptin group compared to increasing the dose of metformin group (48.77 ± 12.73 vs. 50.60 ± 12.36 mg/dl, $P < 0.05$). Serum MDA-LDL levels were not significantly different between the two groups. Statins [3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors] are known to strongly reduce MDA-LDL in type 2 diabetes patients [22] so the influence of interactions between metformin/sitagliptin and statins was also considered. Patients, who are already treated with statins (statin treatment), were not significantly different between two groups (Table 1).

Comparisons of changes of clinical parameters after 6 months in the two groups

Both increasing the dose of metformin and add-on sitagliptin reduced fasting blood glucose (FBG) and HbA1c after 6 months; however, the reduction in FBG was significantly greater in add-on sitagliptin group than in increasing the dose of metformin group (-43.40 ± 70.44 vs. -11.26 ± 57.00 mg/dl, $P < 0.05$). Likewise, the reduction in HbA1c was significantly greater in add-on sitagliptin group than in increasing the dose of metformin group (-1.54 ± 1.50 vs. $-0.69 \pm 1.01\%$, $P < 0.005$). HDL-C was reduced slightly in add-on sitagliptin group and the change in HDL-C was significantly different

TABLE 1. Comparisons of baseline characteristics in increasing the dose of metformin group and add-on sitagliptin group.

	Increasing the dose of metformin group	Add-on sitagliptin group	P value
No. of subjects (male/female)	35 (15/20)	35 (21/14)	NS
Age (years)	60.71±11.01	60.03±12.35	NS
BW (kg)	62.63±13.03	65.29±10.74	NS
BMI (kg/m ²)	25.20±4.52	25.15±3.14	NS
FBG (mg/dl)	208.91±70.18	210.91±73.96	NS
IRI (mU/ml)	10.48±7.93	10.15±9.08	NS
HOMA-IR	5.92±5.31	5.79±6.07	NS
HbA1c (%)	8.75±1.27	9.05±1.69	NS
TC (mg/dl)	196.54±43.42	205.29±39.90	NS
TG (mg/dl)	165.57±87.76	184.09±124.83	NS
HDL-C (mg/dl)	48.77±12.73	50.60±12.36	< 0.05
LDL-C (mg/dl)	117.46±33.92	121.23±34.75	NS
MDA-LDL (U/L)	94.40±37.57	89.94±33.06	NS
Statin treatment (%)	34.2	20.0	NS

Data are presented as mean±SD. BW; body weight, BMI; body mass index, FBG; fasting blood glucose, IRI; immunoreactive insulin, HOMA-IR; homeostasis model assessment-insulin resistance, HbA1c; glycosylated hemoglobin, TC; total cholesterol, TG; triglycerides, HDL-C; high density lipoprotein-cholesterol, LDL-C; LDL-cholesterol, statins; 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. Statin treatment means patients who were already treated with statins.

TABLE 2. Comparisons of the changes in levels of clinical parameters after 6 months of treatment in the two groups

	Increasing the dose of metformin group	Add-on sitagliptin group	P value
ΔBW (kg)	-0.04±2.15	+0.51±1.57	NS
ΔBMI (kg/m ²)	-0.003±0.861	+0.203±0.589	NS
ΔFBG (mg/dl)	-11.26±57.00	-43.40±70.44	< 0.05
ΔIRI (mU/ml)	+0.69±8.60	+1.43±8.87	NS
ΔHOMA-IR	-0.57±6.02	-0.27±5.25	NS
ΔHbA1c (%)	-0.69±1.01	-1.54±1.50	< 0.005
ΔTC (mg/dl)	+0.89±22.26	-8.94±27.62	NS
ΔTG (mg/dl)	+4.37±62.46	+3.46±114.88	NS
ΔHDL-C (mg/dl)	+1.26±4.85	-1.34±5.55	< 0.05
ΔLDL-C (mg/dl)	-0.60±18.40	-4.74±25.00	NS

Data are presented as mean±SD. Abbreviations are as in Table 1.

FIGURE 1. Effects of increasing the dose of metformin and add-on sitagliptin on serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) levels.

(A) MDA-LDL levels before and after six months of increasing the dose of metformin therapy. Data are presented as mean $\pm$ S.E. **P < 0.005 vs. 0 month, paired t-test. (B) MDA-LDL levels before and after six months of add-on sitagliptin therapy. Data are presented as mean $\pm$ S.E. Differences are not statistically significant. (C) Changes in MDA-LDL after six months of increasing the dose of metformin and add-on sitagliptin therapy. Closed bar denotes increasing the dose of metformin group, and open bar denotes add-on sitagliptin group. Data are presented as mean $\pm$ S.E. Δ means the difference between the value at baseline and the value after 6 months. *P < 0.01 vs. add-on sitagliptin group, unpaired t-test.

between two groups (-1.34 ± 5.55 vs. $+1.26 \pm 4.85$ mg/dl, P < 0.05).

HOMA-IR was decreased in both groups. The change in HOMA-IR in increasing the dose of metformin group was slightly stronger than in add-on sitagliptin group; however, the difference between two groups was not significant. Changes in other clinical parameters were not significantly different between two groups (Table 2).

No drug-related adverse effects were observed in any patients.

Change in MDA-LDL in increasing the dose of metformin and add-on sitagliptin groups

MDA-LDL in add-on sitagliptin group after 6 months did not show normal distribution, and the coefficient variation was 0.408, so MDA-LDL was analysed with a logarithmic transformation. Increasing the dose of metformin significantly reduced MDA-LDL levels (mean $\pm$ S.E.) from 94.40 ± 6.35 to 77.83 ± 4.74 U/L (P < 0.005) after 6 months (Figure 1A). In

contrast, add-on sitagliptin resulted in a small, but not statistically significant increase in MDA-LDL from (mean $\pm$ S.E.) 89.94 ± 5.59 U/L to 98.46 ± 6.78 U/L (Figure 1B). The changes in MDA-LDL levels during this study are shown in Figure 1C. The change in MDA-LDL levels (mean $\pm$ S.E) after 6 months of therapy was significantly different between increasing the dose of metformin group and add-on sitagliptin group (-16.57 ± 5.03 vs. $+8.51 \pm 6.90$ U/L, P < 0.01) (Figure 1C).

Multiple regression analysis of association between change in MDA-LDL and various parameters

To determine the factors contributing to the decrease in MDA-LDL, multiple linear regression analysis was performed. Body weight, HOMA-IR and LDL-C were excluded because they had strong interaction with BMI, FBG, IRI and TC, respectively. Results of multiple linear regression analysis between change in MDA-LDL and various clinical parameters are shown in Table 3. Increasing the dose of metformin therapy

TABLE 3. Results of multiple linear regression analysis of association between change in MDA-LDL and various parameters.

Variables	β coefficient	SE	P value
Age	0.097	0.002	0.4492
Gender (0; male, 1; female)	-0.064	0.047	0.6409
Metformin or sitagliptin (0; metformin, 1; sitagliptin)	0.367	0.048	0.0119
Statin treatment (0; no, 1; yes)	0.112	0.048	0.3808
Δ BMI	-0.065	0.029	0.6090
Δ FBG	0.074	0.210	0.6968
Δ IRI	-0.049	0.084	0.7675
Δ HbA1c	-0.121	0.397	0.4621
Δ TC	0.291	0.430	0.0589
Δ TG	-0.031	0.135	0.8134
Δ HDL-C	-0.138	0.004	0.2953

The estimates are adjusted for age, male or female, increasing the dose of metformin or add-on sitagliptin, statin treatment (already treated with statins) or not, Δ BMI, Δ FBG, Δ IRI, Δ HbA1c, Δ TC, Δ TG and Δ HDL-C. Δ refers to the difference between the value at baseline and the value after 6 months. Abbreviations are as in Table 1.

was independently associated with an decrease in MDA-LDL. Other parameters were not significantly related (Table 3). Statins are known to strongly reduce MDA-LDL in type 2 diabetes patients [22]; however, statin treatment (studied in patients already being treated with statins) was not related to a decrease in MDA-LDL in this study.

Discussion

In type 2 diabetes patients receiving on-going metformin treatment, add-on sitagliptin therapy improved FBG and HbA1c to a significantly greater extent compared to simply increasing the dose of metformin. On the other hand, only increasing the dose of metformin decreased MDA-LDL significantly, while add-on sitagliptin had no effect. The decrease in MDA-LDL did not correlate with body weight, glucose or lipid metabolism. Multiple linear regression analysis identified increasing the dose of metformin as the only independent factor associated with change in MDA-LDL levels. Thus, results of the primary outcome measures showed clearly that increasing the dose of metformin, but not add-on sitagliptin, improves MDA-LDL.

Cardiovascular disease is the leading cause of death among type 2 diabetic patients [1]. Metformin, an insulin sensitizer, is more effective than sulfonylurea agents in reducing the incidence of myocardial infarction [5]. This finding may imply that it is important to reduce insulin resistance in patients with type 2 diabetes mellitus.

Sitagliptin, a DPP-4 inhibitor, is known to improve cardiovascular risk factors such as high glucose levels, lipid abnormalities and oxidative stress [15, 17]. Therefore, sitagliptin is expected to reduce atherosclerosis and cardiovascular events. Indeed, a report shows that inhibition of DPP-4 activity reduces atherosclerosis [23].

In this study, both add-on sitagliptin and increasing the dose of metformin reduced blood glucose and HbA1c, but sitagliptin reduced FBG and HbA1c more potently than metformin. In lipid metabolism, increasing the dose of metformin slightly increased HDL-C, while add-on sitagliptin slightly reduced HDL-C. The difference in the change in HDL-C between the two groups was significant. This result implies that increasing the dose of metformin has beneficial effect on HDL-C compared with add-on sitagliptin. These results suggest that increasing the dose of metformin is slightly more beneficial for lipid metabolism and add-on sitagliptin is more potent in improving glucose metabolism compared with the comparator drug in this study.

In this study, we focused on MDA-LDL, which is another cardiovascular risk factor. MDA-LDL is an oxidized LDL and is increased in patients with diabetes or hypertriglyceridemia [8]. Elevated MDA-LDL has been reported to be a risk factor for atherosclerosis or cardiovascular disease [9]. In this study, increasing the dose of metformin significantly reduced MDA-LDL levels while add-on sitagliptin did not change MDA-LDL. The difference in the change in MDA-LDL levels between two groups was significant; therefore, increasing the

dose of metformin was superior to add-on sitagliptin in reducing MDA-LDL in type 2 diabetes patients. Although the changes in HDL-C, fasting blood glucose and HbA1c were significantly different between the two treatment groups, multiple linear regression analysis showed that the change in MDA-LDL levels was not related to the changes in blood glucose, HbA1c or HDL-C.

Increasing the dose of metformin could reduce MDA-LDL levels by a number of mechanisms. First, circulating MDA-LDL levels correlate negatively with peak sizes of LDL particles [10]. A clinical report shows that metformin enlarges LDL particle size in type 2 diabetes patients [11]. The mechanism of the effect of sitagliptin on LDL particle size is unclear. Increased LDL particle size may be one explanation for the reduction in MDA-LDL levels in the increasing the dose of metformin group in this study, but LDL particle size was not evaluated.

Second, some studies have shown that LDL particle size is related to insulin resistance, and troglitazone is known to increase LDL particle size in type 2 diabetes patients [24, 25, 26]. Furthermore, MDA-LDL or MDA levels have also been shown to correlate with insulin resistance [27, 28]. Metformin has been reported to improve insulin resistance [29] and also enlarge LDL particle size [11]. Teneligliptin, which is another DPP-IV inhibitor, has been reported to decrease HOMA-IR, whereas sitagliptin does not change it [30]. In this study, sitagliptin slightly reduced HOMA-IR and the reduction of HOMA-IR in add-on sitagliptin group was weaker than in increasing the dose of metformin group, although the change in HOMA-IR was not significant between two groups. Improvement of insulin resistance may, therefore, be not a reason.

Apolipoprotein B (apoB) is known to be an atherogenic apolipoprotein and is related to small dense LDL-C [31, 32]. Metformin reduces apoB secretion from human hepatocytes and sitagliptin decreases plasma apoB levels in type 2 diabetes patients [33, 34]. Both metformin and sitagliptin have a good effect on apoB, so, we speculated that apoB might not be a mechanism of reduction of MDA-LDL by metformin.

Finally, it has been reported that metformin reduces some oxidative stress markers in human [35, 36] whereas some investigations have reported that sitagliptin improves oxidative stress [37, 38]. However, the comparative degree of antioxidant effect between these two drugs is unknown and the antioxidant effect of metformin may be stronger than that of sitagliptin.

Although four possibilities were considered as to why increasing the dose of metformin lead to reduced MDA-LDL levels in this study, LDL particle size, apoB or other oxidative stress markers were not evaluated. Thus, the mechanism by

which metformin improved MDA-LDL levels requires further study.

There are some limitations in the present study. First, 35 patients were investigated in each treatment group, which is a relatively small in number. Second, the two groups were not completely matched in patient background: HDL-C was significantly higher in add-on sitagliptin group. However, the change in HDL-C was found not to be related to the change in MDA-LDL. Third, the study duration was only 6 months, so the long-term effects of increasing the dose of metformin and add-on sitagliptin are unclear.

Although there are some limitations to the study, the results of the primary end points, which are serum MDA-LDL levels and changes in MDA-LDL levels after 6 months of treatment, clearly show that increasing the dose of metformin lowers MDA-LDL and the regression study identified increasing the dose of metformin as the only independent factor associated with reduced MDA-LDL level. The metformin-induced change in MDA-LDL levels appears to contribute to the drug's ability to prevent cardiovascular disease.

In summary, increasing the dose of metformin therapy in patients with type 2 diabetes reduced serum MDA-LDL levels, an oxidized LDL associated with atherosclerosis. This effect was not observed with add-on sitagliptin therapy. These results suggest that metformin decreases MDA-LDL in type 2 diabetes mellitus patients and this effect may be a mechanism by which metformin prevents cardiovascular disease.

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