Association between Serum Ferritin and Circulating Oxidized Low-density Lipoprotein Levels in Patients with Type 2 Diabetes

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Abstract. The oxidative modification of low-density lipoproteins (LDL) plays a central role in the initiation and acceleration of atherosclerosis. Iron plays a part in the formation of highly toxic free radicals such as hydroxide and superoxide anions, which can induce lipid peroxidation. We investigated whether serum iron status was associated with circulating oxidized LDL (oxLDL) levels in type 2 diabetic patients, in whom oxidative stress and susceptibility to lipid oxidation were supposedly increased. Serum ferritin levels were significantly correlated with plasma oxLDL concentrations in both male and female patients ($p < 0.02$ and $p < 0.05$, respectively). No correlation was detected between ferritin and LDL-cholesterol (LDL-C) concentrations despite the close correlation between LDL-C and oxLDL concentrations ($p < 0.0001$). Stepwise regression analysis showed that ferritin concentration was an independent positive determinant of oxLDL level, in addition to triglyceride concentration, body mass index and sex. This is the first report to show that serum ferritin is associated with circulating oxLDL levels in patients with type 2 diabetes. Further work is required to establish a causative link between iron excess and the development of diabetic vascular complications.

Key words: Oxidized LDL, Iron, Ferritin, Type 2 diabetes

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THE oxidative modification of low-density lipoproteins (LDL) plays a central role in the initiation and acceleration of atherosclerosis [1, 2]. Oxidized LDL (oxLDL), which is generally considered to be generated in vessel walls, exerts several proatherogenic effects, including increased synthesis and secretion of adhesion molecules, monocyte chemotaxis and adhesion, cytotoxicity to endothelial cells, enhanced foam cell formation, and increased smooth muscle cell proliferation [3, 4]. oxLDL has also been identified in the circulation and has been shown to be associated with coronary artery disease [5, 6], although it remains unclear whether the plasma oxLDL originates in the arte-

rial wall or is generated in the circulation.

It is well known that iron is closely associated with oxidative stress. Iron takes part in the formation of highly toxic free radicals such as hydroxide and superoxide anions, which can induce lipid peroxidation [7]. Materials from atherosclerotic plaques have been shown to both contain iron and stimulate hydroxyl-radical generation and lipid peroxidation [8, 9]. In accordance with this, it has been reported that iron chelation blocks oxidation of LDL [10]. Unused iron is stored as ferritin molecules to ensure neutralization, and iron must be in its free form to act as a prooxidant agent. Iron can be released from ferritin by the action of a reducing agent that converts Fe^{3+} into Fe^{2+} , and its release is accelerated when antioxidant concentrations are decreased [7]. Thus, ferritin can act both as a molecule that protects against iron toxicity and as a source of iron that induces oxidative stress [11].

Considerable evidence suggests that hyperglycemia is associated with excess oxidation [12, 13]. It has been

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shown that iron-induced LDL oxidation is enhanced by glucose in a time- and concentration-dependent manner *in vitro* [14]. Hyperglycemia is also associated with mild but chronic inflammation, which can accelerate the oxidation of lipoproteins [15]. Further, non-enzymatic glycation of LDL makes it more susceptible to oxidation [16]. Despite the accumulated data on the relation between iron, hyperglycemia and lipoprotein oxidation *in vitro*, there is no data showing an association between iron status and oxLDL in the diabetic state. We therefore studied the relationship between circulating oxLDL levels and ferritin concentrations in patients with type 2 diabetes.

Materials and Methods

We recruited 144 patients with type 2 diabetes based on the 1999 World Health Organization criteria (74 males and 70 females; mean age $\pm$ SD: 64 ± 12 years). These patients had no ketoacidosis, renal failure (serum creatinine ≥ 2.0 mg/dL), liver disorders, or recent history of cardiovascular disease. Subjects being treated with iron or drugs known to affect lipoprotein oxidation (*e.g.* vitamin E, vitamin C, or probucol) were excluded from the study. Subjects who had any history of blood transfusion were also excluded. None of the subjects had excessive alcohol intake. All subjects were Japanese and resided in the same area (Kochi Prefecture, Japan), and gave written informed consent to participate prior to the study.

Patients were considered to have diabetic nephropathy if they had a urine protein concentration of >0.30 g/L at two or more consecutive measurements separated by an interval of 4 weeks or more. Patients were considered to have coronary artery disease if they had any one of the following: 1) acute myocardial infarction or confirmed non-acute myocardial infarction based on serial readings of baseline and annual electrocardiograms; 2) coronary artery disease requiring bypass surgery or angioplasty; or 3) angina confirmed by angiography or by ischemic changes found during non-invasive testing.

Fasting blood samples were collected from the subjects and transferred to tubes with or without EDTA-2Na for plasma and sera, respectively. Plasma and sera were immediately isolated by centrifugation and stored frozen at -80°C until use. Plasma oxLDL concentration was measured by a sandwich enzyme-linked

immunosorbent assay using monoclonal anti-oxidized LDL antibody (mAb-4E6) [5] and anti-apolipoprotein B antibody (Oxidized LDL ELISA kit, Mercodia AB, Uppsala, Sweden). Serum ferritin concentration was determined using commercially available kits (ECLusys Ferritin, Roche Diagnostics, Basel, Switzerland). High sensitive C-reactive protein (hsCRP) concentration was measured using latex-enhanced immunonephelometry (N High Sensitivity CRP, Dade Behring, Deerfield, IL, USA).

All data are presented as the mean $\pm$ SD. Comparisons of variables between two groups were performed using the Student's unpaired *t*-test. Correlations between different parameters were determined by Pearson product-moment correlation coefficients. Because serum ferritin concentrations showed skewed distributions in both sexes they were log-transformed (log-transformed ferritin). Multiple regression analysis was conducted using a stepwise method and a software program on a personal computer (StatView, version 4.5; Abacus Concepts, Berkeley, CA, USA). Sex (male = 1, female = 2), age, body mass index (BMI), duration of diabetes, $\text{HbA}_{1\text{C}}$, log-transformed ferritin, hemoglobin, triglycerides, high density lipoprotein (HDL)-cholesterol, uric acid, hsCRP, hypertension (absent = 1, existing = 2), current smoking status (no = 1, yes = 2), and statin use (no = 1, yes = 2) were incorporated as independent variables for analysis of plasma oxLDL concentration. Variables were considered independent determinants if the *F* value was confirmed to be more than 4.0. Statistical significance was defined as $p < 0.05$.

Results

The mean plasma oxLDL concentration in the study population was 51.5 ± 15.3 U/L (range 19.6–95.0 U/L), and it was significantly higher in female patients than male patients (Table 1). Serum ferritin concentration was significantly higher in male patients than female patients (225.4 ± 163.3 vs 95.6 ± 97.3 ng/mL, $p < 0.0001$, respectively) (Table 1). Log-transformed ferritin concentration was significantly correlated with plasma oxLDL concentration in both male and female patients (Fig. 1-a). No correlation was detected between log-transformed ferritin concentration and LDL-cholesterol (LDL-C) concentration (Fig. 1-b), although the LDL-C concentration was closely correlated with oxLDL concentration in both male ($r = 0.664$, $p < 0.0001$) and fe-

Table 1. Clinical characteristics of the study subjects

Parameter	Male	Female	<i>p</i>
<i>n</i>	74	70	
Age (years)	63.6 ± 12.5	64.7 ± 12.2	N.S. ^a
BMI (kg/m ²)	24.3 ± 5.4	25.1 ± 5.3	N.S. ^a
Diabetes duration (years)	14.8 ± 9.1	13.8 ± 8.4	N.S. ^a
HbA _{1c} (%)	7.0 ± 1.1	7.5 ± 1.4	0.0173 ^a
Total cholesterol (mg/dL)	174 ± 40	198 ± 35	0.0002 ^a
Triglycerides (mg/dL)	129 ± 77	130 ± 72	N.S. ^a
HDL-cholesterol (mg/dL)	47 ± 13	54 ± 17	0.0099 ^a
LDL-cholesterol (mg/dL)	101 ± 36	119 ± 30	0.0015 ^a
Hemoglobin (g/dL)	13.3 ± 2.0	12.4 ± 1.7	0.0035 ^a
Creatinine (mg/dL)	0.91 ± 0.53	0.79 ± 0.52	N.S. ^a
Uric acid (mg/dL)	5.5 ± 1.4	4.7 ± 1.4	0.0007 ^a
High sensitive CRP (mg/dL)	0.30 ± 0.71	0.22 ± 0.34	N.S. ^a
Therapy (diet/OHA/insulin)	14/38/22	7/26/37	0.0158 ^b
Statin use	12 (16.2%)	30 (42.9%)	0.0004 ^b
Hypertension	43 (58.1%)	44 (62.9%)	N.S. ^b
Current smoking	47 (63.5%)	14 (20.0%)	<0.0001 ^b
Persistent proteinuria	17 (23.0%)	12 (17.1%)	N.S. ^b
Coronary artery disease	14 (18.9%)	15 (21.4%)	N.S. ^b
Oxidized LDL (U/L)	48.3 ± 14.3	54.8 ± 15.7	0.0101 ^a
Ferritin (ng/mL)	225.4 ± 163.3	95.6 ± 97.3	<0.0001 ^a

Data are presented as number (percentage) or mean ± SD. Statistical differences between males and females were tested using an unpaired Student's *t* test^a or a chi-square test^b. N.S.: not significant. Hypertension: diastolic blood pressure ≥90 mmHg, systolic blood pressure ≥140 mmHg, and/or use of antihypertensive medication. OHA: oral hypoglycemic agent.

male patients ($r = 0.525$, $p < 0.0001$). Log-transformed ferritin concentration also showed a significantly positive correlation to oxLDL levels adjusted for serum LDL-C concentration (oxLDL/LDL-C ratio) in male ($r = 0.258$, $p < 0.05$) but not in female patients ($r = 0.081$, not significant). Stepwise regression analysis of the independent determinants for oxLDL concentration showed that log-transformed ferritin concentration was a positive determinant of oxLDL concentration in addition to triglycerides, sex and BMI (Table 2-a). If we incorporated LDL-C concentration as an independent variable into this model, only LDL-C ($\beta = 0.606$, $F = 99.083$) and triglyceride concentrations ($\beta = 0.364$, $F = 35.658$) remained significant for oxLDL concentration (total $R^2 = 0.511$, $p < 0.0001$ for the model) because of the close relationship between oxLDL and LDL-C concentrations. Another stepwise regression analysis in which the oxLDL/LDL-C ratio was used as a dependent variable instead of oxLDL also showed log-transformed ferritin to be an independent contributor, as were triglycerides and hemoglobin concentration (Table 2-b).

Table 2. Stepwise multiple regression analysis for plasma oxLDL concentration (a) or oxLDL/LDL-C ratio (b) in diabetic patients

Variable	β	<i>F</i>	<i>p</i>
(a) oxLDL concentration			
Triglycerides	0.299	15.434	0.0001
Sex	0.317	13.836	0.0003
BMI	0.247	10.642	0.0014
Log ferritin	0.233	6.605	0.0113
(b) oxLDL/LDL-C ratio			
Triglycerides	0.381	23.808	<0.0001
Hemoglobin	-0.232	8.705	0.0034
Log ferritin	0.180	5.063	0.0261
Hypertension	0.159	3.376	0.0684
High sensitive CRP	0.154	3.173	0.0772

Stepwise multivariate regression analysis was performed. Parameters that were incorporated as independent variables were as follows: sex (male = 1, female = 2), age, BMI, duration of diabetes, HbA_{1c}, log-transformed ferritin, hemoglobin, triglycerides, HDL-cholesterol, uric acid, high sensitive CRP, hypertension (absent = 1, existing = 2), smoking (no = 1, yes = 2), statin use (no = 1, yes = 2).

The *F* value for the inclusion and exclusion of variables was set at 4.0 at each step. β : partial regression coefficient.

(a) Total $R^2 = 0.300$, $p < 0.0001$ for the model. (b) Total $R^2 = 0.228$, $p < 0.0001$ for the model.

Female patients with persistent proteinuria had significantly higher ferritin levels compared to those without (146.4 ± 143.9 vs 85.1 ± 82.5 ng/mL, respectively, $p < 0.05$) although the difference did not reach significance in male patients (258.5 ± 194.9 vs 215.5 ± 153.3 ng/mL, respectively). There was no difference in ferritin levels between patients with and without coronary artery disease in both sexes (data not shown).

Discussion

Tissue iron excess has been shown to contribute to the production of reactive oxygen species and oxidative tissue damage. Body iron stores, expressed as serum ferritin levels, have been suggested to be associated with atherosclerosis [17, 18], although the epidemiological data are still conflicting [19]. The most widely accepted hypothesis explaining these associations is that a high ferritin level is associated with higher oxidative stress, which can accelerate the oxidation of lipoproteins. In the present study, we found significant positive correlations between serum ferritin concentrations and circulating oxLDL levels in both male

Fig. 1. Correlations between log-transformed ferritin and plasma oxidized LDL (a) and LDL-cholesterol (b) concentrations in type 2 diabetic patients.

Single linear univariate correlations were evaluated by Pearson's correlation coefficient.

and female patients with type 2 diabetes. The oxLDL level was closely correlated with LDL-C concentration; however, no significant correlation was observed between ferritin and LDL-C levels. Further, multiple regression analysis for oxLDL confirmed that ferritin concentration was a significant independent contributor, as were sex, BMI, and triglycerides, which are well-known risk factors for atherosclerosis. This is the first report showing a positive association between serum ferritin and circulating oxLDL levels in diabetic patients, in whom oxidative stress and susceptibility to lipid oxidation are supposedly increased.

There are a few clinical reports in which associations between ferritin levels and LDL oxidation parameters have been examined [20, 21]. For example, Iribarren *et al.* [20] reported that ferritin concentrations were not associated with susceptibility to LDL oxidation (dura-

tion of the lag phase after inducing oxidation with hemin) or autoantibodies against malondialdehyde (MDA)-modified LDL in a general population including 8–10% diabetic patients. On the other hand, Brouwers *et al.* [21] found that serum ferritin concentration was independently associated with plasma MDA concentration and the oxLDL/LDL-C ratio, which may reflect the proportion of oxidatively modified LDL to total LDL, in healthy male but not female Caucasian subjects. This result seems to be consistent with our present result, although Brouwers *et al.* did not demonstrate a direct association between serum ferritin concentration and circulating oxLDL level. Also, among our male patients log-transformed ferritin concentration was positively correlated with oxLDL/LDL-C ratio. In addition, multiple regression analysis confirmed that log-transformed ferritin was an independent con-

tributor to the oxLDL/LDL-C ratio as well as oxLDL concentration. It remains unclear why both the present findings and those of Brouwers *et al.* did not show a significant association between oxLDL/LDL-C ratio and serum ferritin levels in female subjects. Among our patients, triglycerides and HDL-cholesterol concentrations, both of which are major components of the metabolic syndrome, were correlated with the oxLDL/LDL-C ratio more closely in females ($r = 0.528$, $p < 0.0001$ and $r = -0.460$, $p < 0.0001$, respectively) than males ($r = 0.289$, $p < 0.05$ and $r = -0.104$, not significant, respectively). Such differences in the impacts of other contributing factors on the oxLDL/LDL-C ratio could be part of the reason for the different associations between serum ferritin levels and the oxLDL/LDL-C ratio between sexes.

There are some reports suggesting a significant influence of tissue iron excess on the development of diabetic nephropathy and vascular dysfunction [22, 23]. In the present study, serum ferritin levels in patients

with nephropathy were higher than in patients without. Although ferritin is a source of iron that induces oxidative stress, it can also act as a molecule that protects against iron toxicity by neutralizing unused free iron [11]. Because iron can be released from ferritin by the action of a reducing agent that converts Fe^{3+} into Fe^{2+} , its release is accelerated when antioxidant concentrations are decreased [7]. In diabetic patients who are thought to have increased oxidative stress and decreased antioxidant capacity, ferritin may act as an undesirable molecule because iron release from ferritin is promoted under these conditions.

It should be noted that this was a cross-sectional study and that the number of subjects participating was too small to arrive at definitive conclusions. Therefore, a large-scale prospective study is required to establish a link between serum ferritin concentration and the development of vascular complications in diabetic patients.

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