Copper-Modified Low Density Lipoprotein from Diabetic Patients Enhances Cholesteryl Ester Synthesis by Mouse Peritoneal Macrophages

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Summary In this study, the capability of superoxide production by LDL either without stimulation or under the stimulation of Fe-ADP and the manner of modification of low density lipoprotein (LDL) by copper (Cu^{++}) in diabetic patients were examined and compared with those of healthy subjects. The amount of superoxide generated by diabetic LDL was similar to that of healthy subjects both at the basal level and following stimulation by Fe-ADP. LDL from healthy subjects and diabetic patients was modified in the presence of $10\ \mu\text{M}$ CuSO_4 . When the level of thiobarbituric acid-reactive substance was determined in the Cu^{++} -modified LDL, no difference was found. On the other hand, when the incorporation of [^{14}C]oleate into cholesteryl ester (CE) by mouse peritoneal macrophages was stimulated by the Cu^{++} -modified LDL, we found that the modified diabetic LDL significantly enhanced the CE synthesis compared with the modified healthy LDL ($p < 0.01$). To clarify the mechanism of the enhanced CE synthesis, the rate of degradation of [^{125}I] Cu^{++} -modified diabetic LDL was measured. We found that this rate was similar to that of healthy subjects. These data suggest that the degree of oxidation does not differ among patients although diabetic LDL is modified by Cu^{++} in a manner such that it preferentially promotes the activity of CE synthesis in macrophages by some unknown intracellular metabolic mechanism.

Key Words: diabetes, superoxide, LDL, foam cell, macrophages

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The incidence of atherosclerosis is high in diabetic patients [1-3], and the reason for this is not clear. However, it has been speculated that various atherogenic factors work cooperatively or synergistically to promote the development of atherosclerosis.

In the present study, two specific atherogenic factors were focused upon. The first is the superoxide generated by diabetic LDL, because it has been reported that the free radical accelerates the development of atherosclerosis in several ways [4]. The second is the manner of modification of low density lipoprotein (LDL). Modified LDL is involved in the deposition of cholesteryl ester (CE) in macrophages [5-7]. Macrophages are thought to be the precursors of most of the lipid-laden foam cells characteristic of the early atheromatous lesion [8, 9], and CE synthesis is associated with the deposition of CE in macrophages [5]. Our results indicate that diabetic LDL is modified by Cu^{++} in a unique manner whereby it significantly enhances the synthesis of CE by macrophages.

MATERIALS AND METHODS

Subjects. Two groups of subjects were studied after informed consent had been obtained: 5 healthy individuals comprising 2 males and 3 females (healthy group) and 11 diabetic patients comprising 7 males and 4 females (diabetic group). All diabetic patients except one had non-insulin-dependent diabetes mellitus and were diagnosed based upon the criteria of the National Diabetes Data Group concerning the 75-g oral glucose tolerance test [10]. Blood glucose of diabetic patients was controlled with a stable treatment regimen for 4 months before the experiments, and glucose levels were stable at the time of blood withdrawal. Five patients in the diabetic group were treated with insulin, and the other 6 received oral hypoglycemic agents. None of the diabetic patients had complications of atherosclerosis such as ischemic heart disease or cerebral vascular disease. Six of the 11 diabetic patients had hypertriglyceridemia (plasma triglyceride > 1.69 SI units).

Low density lipoprotein separation. Following an overnight fast, 20 ml blood was withdrawn from each subject. Ethylenediaminetetraacetic acid (EDTA) was added to obtain a final concentration of 1 mg/ml. Low density lipoprotein (LDL) ($1.019 < d < 1.063$) was separated by ultracentrifugation [11]. Protein was measured by the method of Lowry *et al.* [12].

Superoxide generation from LDL. EDTA was first removed from the LDL by dialysis against 10 mM sodium phosphate buffer (pH 7.4) (PBS) containing 150 mM NaCl. Measurement of superoxide was performed at the basal level and following the stimulation by Fe-ADP in five normal LDL samples and five diabetic LDL samples. The experiment was performed in the dark room and finished within 4 h. Employing one LDL sample, the basal photon level was followed from the beginning of the experiment until the end. The value of the basal photon count did not alter during 4 h of the experiment. The superoxide

generation was determined by the 2-methyl-6-phenyl-3,7-dihydroimidazo [1, 2 α] pyrazin-3-one (MCLA)-dependent chemiluminescence method [13]. The system containing 4 μ M MCLA and 100 μ g LDL (final concentration: 33 μ g/ml of LDL protein) in 3 ml of 10 mM HEPES (pH 7.4) was incubated for 5 min at 37°C, and chemiluminescence was detected. The addition of 8.6 μ M Fe-ADP complex to the system caused an immediate increase in the photon count; this increase could be completely inhibited by 50 units of superoxide dismutase (SOD). The level of SOD-inhibited photon count was used for the estimation of both the basal and Fe-ADP complex-stimulated superoxide release from LDL.

Modification of LDL by CuSO₄. To determine the susceptibility of LDL to modification, we used LDL from which EDTA had been eliminated by dialysis. LDL was then oxidized in the presence of 10 μ M CuSO₄ for 12 h by the method of Heinecke *et al.* [14]. Lipid peroxide formation was estimated by the measurement of thiobarbituric acid-reactive substances (TBARS) according to the method of Schuh *et al.* [15]. The optical density of the supernatant was determined at 532 nm and was compared with that of a freshly prepared malondialdehyde (MDA) standard [16]. The results were expressed as MDA equivalents per milligram of LDL protein. The oxidized LDL was dialyzed in RPMI 1640 and used in the following procedure.

[¹⁴C]oleate incorporation into cholesteryl ester by mouse peritoneal macrophages. Resident peritoneal cells from unstimulated mice were harvested in PBS as described by Edelson and Cohn [17]. The cells were plated in 35-mm wells at a concentration of 3×10^6 cells/well and were cultured for 18 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Then the culture medium was removed and the adherent macrophages were washed twice with RPMI 1640. One milliliter of RPMI 1640 was added and cells were cultured for 6 h in the presence of both 20 nmol [¹⁴C]oleate (NEN Research Products, Boston, MA) and 50 μ g of modified LDL. After incubation, the medium was removed and the cells were washed twice with PBS. Lipids were extracted with hexane and isopropanol (3 : 2, v/v). After the solvent had been evaporated under N₂ gas, the extracted lipid was separated by silica gel G thin-layer chromatography. The chromatogram was developed in heptane : diethyl ether : acetic acid (90 : 10 : 1). The CE spot was identified by use of iodine vapor and was cut from the chromatogram. Scintillation solution (Aquasol 2, NEN Research Products) was added, and the radioactivity was counted in a liquid scintillation counter. Correction for procedural losses was made by the addition of [³H]cholesteryl oleate (NEN Research Products) to the well prior to the lipid extraction. One milliliter of 0.2 M NaOH was added to the well in which cells remained for the measurement of cell protein [12]. Triplicate dishes of cells were used for each LDL sample.

Metabolism of [¹²⁵I]-copper-modified normal LDL and [¹²⁵I]-copper-modified diabetic LDL. Three samples each were chosen at random from 5 Cu⁺⁺-modified normal LDL and 5 Cu⁺⁺-modified diabetic LDL that were prepared as described above. Those six samples were labeled with [¹²⁵I] by the iodine

monochloride method [18]. Mouse peritoneal macrophages were prepared as described above. [^{125}I]-labeled Cu^{++} -modified normal LDL or [^{125}I]-labeled Cu^{++} -modified diabetic LDL was added at a concentration of $50\text{ }\mu\text{g/ml}$, and the amounts of degradation and cell association were measured after a 6-h incubation as described previously [19].

Miscellaneous analysis. Fasting plasma glucose, total cholesterol, triglyceride, and hemoglobin $\text{A}_{1\text{c}}$ were measured in the Tokai University Hospital Central Laboratory by commonly used procedures.

Statistical methods. Comparisons between the groups were made by the Mann-Whitney non-parametrical statistical analysis [20]. Correlation coefficients were determined by a standard statistical method.

RESULTS

The diabetic group had a higher fasting plasma glucose level accompanied by a higher level of hemoglobin $\text{A}_{1\text{c}}$ than the healthy group (Table 1). The amount of superoxide generated from LDL was measured both at the basal level and following the stimulation by Fe-ADP. The photon count increased approximately ten-fold following the stimulation by Fe-ADP (Table 2). When the photon count of the healthy and diabetic groups was compared, no significant difference was observed either at the basal level or after the Fe-ADP stimulation. The levels of TBARS were measured pre and post modification by Cu^{++} . The level of TBARS in LDL following Cu^{++} modification was more than the original value without Cu^{++} modification (Table 3). When the level of TBARS between the two groups was compared, no significant difference was observed either before or after Cu^{++} modification. When the TBARS levels of each sample following Cu^{++} modification were plotted against the original TBARS values without modification, no significant correlation was observed. When the effect of Cu^{++} -modified LDL on the activity of CE synthesis by macrophages was tested, we found that the CE

Table 1. Plasma glucose and $\text{HbA}_{1\text{c}}$ in healthy subjects and diabetic patients.

Group	<i>n</i>	Glucose (SI units)	$\text{HbA}_{1\text{c}}$ (%)
Healthy	5	4.9 (4.5–5.5)	5.1 (4.8–5.3)
Diabetic	11	10.4 (5.2–17.4)*	8.1 (7.3–12.4)**

Values are median with ranges in parentheses. Normal range of hemoglobin $\text{A}_{1\text{c}}$ ($\text{HbA}_{1\text{c}}$) is 5.2–6.7%. * $p < 0.001$, ** $p < 0.0009$, diabetic vs. healthy group.

Table 2. Photon count of LDL.

	Healthy subjects	Diabetic patients
Number	5	5
Basal photon count	10 (10–20)	9 (6–11)
Photon count stimulated by Fe-ADP	160 (94–177)	131 (91–165)

Values are median with ranges in parentheses.

Table 3. TBARS in LDL with and without modification by Cu⁺⁺.

Group	<i>n</i>	TBARS in LDL	
		Pre-modification (nmol/mg protein)	Post-modification (nmol/mg protein)
Healthy	5	1.33 (1.15–1.71)	31.35 (19.56–59.80)
Diabetic	11	1.58 (1.36–2.06)	38.23 (23.63–69.45)

Values are median with ranges in parentheses.

Table 4. Cholesteryl ester synthesis by macrophages in the presence of copper-modified LDL.

Group	<i>n</i>	[¹⁴ C]oleate incorporation into cholesteryl ester (nmol/mg of cellular protein/6 h)
Healthy	5	1.89 (1.41–3.06)
Diabetic	11	3.42 (2.01–7.05)*

Values are median with ranges in parentheses. **p* < 0.01, healthy vs. diabetic group.

Table 5. Degradation and internalization of [¹²⁵I]copper-modified LDL by macrophages.

Source	<i>n</i>	Degradation (μg/mg protein/6 h)	Cell association (μg/mg protein/6 h)
Cu ⁺⁺ healthy LDL	3	9.53 ± 1.44	0.013 ± 0.001
Cu ⁺⁺ diabetic LDL	3	9.49 ± 0.66	0.013 ± 0.001

All values are expressed as mean ± SD.

synthesis was significantly higher in the cells treated with modified diabetic-group LDL than in those treated with the healthy-group modified LDL (*p* < 0.01) (Table 4). No significant correlation was obtained between CE synthesis by macrophages and TBARS levels in Cu⁺⁺-modified LDL. The relationship of the enhanced CE synthesis by Cu⁺⁺-modified diabetic LDL and the HbA_{1c} level was also analyzed; however, no significant relationship was found.

To clarify the mechanism of the enhanced CE synthesis in the presence of Cu⁺⁺-modified diabetic LDL, we examined the metabolism of apoprotein B in Cu⁺⁺-modified diabetic LDL by macrophages. Cu⁺⁺-modified diabetic LDL was labeled with [¹²⁵I], and the levels of internalization and degradation were measured and compared with those of [¹²⁵I]-labeled Cu⁺⁺-modified normal LDL. The levels of both degradation and cell association were found to be similar (Table 5).

Since 6 diabetic patients had hypertriglyceridemia, a comparison study was also performed in all parameters described above between the two diabetic groups with and without hypertriglyceridemia. No significant difference was observed.

DISCUSSION

The present study was undertaken to determine the atherogenic factors that are unique to diabetes mellitus. Two atherogenic factors were examined, the first

being the capability of superoxide production by diabetic LDL. Since it has been reported that the free radical accelerates the development of atherosclerosis in several ways [4], we attempted to measure the superoxide generation in patients in whom atherosclerosis is common. We found that monocytes generate a greater amount of superoxide in those patients [21] and that the superoxide generated by monocytes is partially responsible for the oxidation of LDL [22]. However it still remains unclear as to whether or not LDL alone is capable of generating superoxide. Recently, Sakurai *et al.* [13] reported that glycated polypeptide generates superoxide and that this action is accelerated by the presence of iron. Therefore, we tested the possibility that glycosylated apoprotein B of LDL in diabetes produces a greater amount of superoxide. We found that diabetic LDL and healthy LDL generate superoxide to a similar extent. A possible reason is that the degree of glycosylation in diabetic LDL *in vivo* [23] seems insufficient compared with that of the *in vitro* system [13].

The second purpose of the present study was to investigate the manner of LDL-modification by copper. The activity of CE synthesis by macrophages in the presence of Cu^{++} -modified diabetic LDL was increased independent of the level of TBARS. LDL oxidation by Cu^{++} and metabolism by macrophages have been reported by Steinbrecher *et al.* [24]. Cu^{++} -modified LDL is taken up by scavenger receptors in macrophages and digested in lysosomes; and the hydrolyzed free cholesterol is re-utilized for the synthesis of new CE. This research group showed that the degree of oxidation in LDL had a linear relationship with both the amount of the degradation of [^{125}I]-labeled Cu^{++} -modified LDL and CE synthesis by macrophages. In that system, LDL's oxidized to various degrees were prepared from healthy LDL from a single source by alteration of the experimental conditions. In contrast, in the present study, there was no direct correlation between TBARS and CE synthesis. A possible explanation is as follows: The measurement of TBARS may be a very crude method of assessment of oxidation and may not precisely monitor the changes critical to determining cellular CE synthesis, as Jialal *et al.*, have already reported [25]. This wide variation may make it difficult to analyze the relationship between LDL oxidation and CE synthesis.

In order to clarify the mechanism of enhancement of CE synthesis by the Cu^{++} -modified diabetic LDL, we examined the metabolism of apoprotein B of Cu^{++} -modified diabetic LDL. Regarding the metabolism of glycosylated LDL without Cu^{++} modification, it remains controversial as to whether the glycosylated LDL alone is capable of enhancing the CE synthesis. The LDL that is extensively glycosylated *in vitro* has been shown to enhance the CE synthesis by human monocyte-derived macrophages [26] but not that by murine macrophages [27, 28]. Therefore, in the present study employing mouse peritoneal macrophages, the modification by Cu^{++} was added to diabetic LDL, and this procedure successfully led to the enhancement of CE synthesis. When the trichloroacetic-acid-soluble products were measured, indicating the degradation of [^{125}I]-labeled apoprotein in Cu^{++} -modified diabetic LDL, the amount of degradation was the same as that of

Cu⁺⁺-modified normal LDL. This leads us to speculate that some alteration in the lipid part may be responsible for the enhanced CE synthesis. It has been reported that CE synthesis is increased by 7-ketocholesterol [29] and decreased by 7-hydroxycholesterol [30]. Thus there remains the possibility that oxidized diabetic LDL may contain a different mixture of oxidized lipids whose net effect may stimulate the CE synthesis more. As another possibility, the CE content of oxidized LDL may be decreased following the procedure of oxidation. It may possibly be decreased less in oxidized diabetic LDL than in oxidized normal LDL, thus leading to apparently greater CE synthesis with oxidized diabetic LDL. To assess these possibilities, further study is required.

The level of HbA_{1c} is known to correlate with the degree of glycosylation of LDL [23]; however, the CE synthesis was not significantly related to the level of HbA_{1c}. Thus, the extension of glycosylation does not account for the enhancement of CE synthesis; however, glycosylation does seem to be a minimum requirement.

In the present experimental system, Cu⁺⁺ was employed to produce free radicals. However, it has also been shown that free radicals are released from several types of cells that constitute the arteries, such as endothelial cells [31] and arterial smooth muscle cells [14]. Thus, diabetic LDL may be modified by these cells *in vivo*, leading to the higher level of CE deposition in macrophages. In a previous serial study to determine disease-specific atherogenic factors, several factors were found to be unique to hypertriglyceridemia [19, 21, 32]. However, in the present study we found that the manner of LDL modification by Cu⁺⁺ was independent of hypertriglyceridemia.

We conclude that the altered manner of LDL modification in diabetes mellitus may accelerate the development of atherosclerosis, working cooperatively or synergistically with other previously known atherogenic factors [33].

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