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OXIDATIVE MODIFICATION OF GLYCATED LOW DENSITY LIPOPROTEIN IN THE PRESENCE OF IRON

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This is the first observation for contributing to the glycation of low density lipoprotein (LDL) to oxidative modification of its own lipids and protein. Human plasma LDL was glycated by incubation with glucose (G-LDL). Glucose incorporated into apoprotein B was approximately 10 mol/mol of apoprotein (2.8 % modification of lysine residues) and 84 % of G-LDL was adsorbed on phenylboronate affinity column. G-LDL incubated with Fe^{3+} for 4 h caused a significantly higher level of lipid peroxidation than U-LDL (untreated with glucose), and a higher molecular weight protein was observed in apoprotein B on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), increasing with incubation period. Corresponding to change on SDS-PAGE, G-LDL exposed to Fe^{3+} moved faster than G-LDL per se or U-LDL to anode on agarose gel electrophoresis. The higher the Fe^{3+} concentration, the more lipid peroxidation was caused. α-tocopherol or probucol suppressed the lipid peroxidation of G-LDL exposed to Fe^{3+}.

Much attention has been focused on the role of modified low density lipoprotein (LDL) in the development of atherosclerosis. Chemically acetylated LDL could be recognized by scavenger receptors of monocyte-macrophages, but not by LDL receptors of cells (1). It is widely accepted that endocytosis of LDL by macrophages results in the formation of foam cells. Oxidized LDL has been also reported to be mediated by scavenger receptors and to form lipid-laden foam cells (2). Oxidation of LDL in

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Abbreviations: LDL, low density lipoprotein; TBARS, thiobarbituric acid reacting substances; ADP, adenosine 5'-diphosphate sodium salt.

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vitro studies is usually initiated by low concentration of cuppric ion. However, what and where in vivo does causes the oxidation of LDL? Evidences of presence of oxidized LDL in vivo were shown in aorta of WHHL rabbits by immunocytochemistry, which clearly demonstrated the extracellular deposition of apoprotein B modified by lipid peroxidation products (3). Naturally occurring oxidants in the circulation, however, are unclear except for endothelial cell's lipoxygenase (4). Recently, we reported that non-enzymatic glycation of polypeptide chelates with iron and generates superoxide anion (5). The glycated polylysine chelated with ferric ion elicits lipid peroxidation of phospholipid liposome from rat liver microsome and human LDL at physiological pH (6,7). These results urged us to investigate the effects of glycation of LDL on its own lipids.

MATERIALS AND METHODS

Preparation of LDL. LDL ($1.019 < d < 1.063$) was isolated by ultracentrifugation from human plasma which contained 0.4 mg of 2 Na EDTA/ml, according to the method described by Schumaker and Puppione (8). The isolated LDL was dialyzed against 67 mM phosphate buffer(pH 7.4) saturated with N_2 gas. Protein concentration was determined by Lowry's method. Purity of LDL was verified by agarose gel (1%) electrophoresis, SDS-polyacrylamide gel (10%), and immunoassay of apoprotein. LDL sample showed single band on both electrophoretic plate after staining with coomassie brilliant blue staining (for SDS-polyacrylamide gel plate) and Fat Red 7B staining (for agarose gel plate), and it contained about 0.7 mg of apoprotein B/mg of protein.

Glycation of LDL. LDL (2mg of protein) in 2 ml of 67 mM phosphate buffer containing 200 mM glucose and 0.01 % gentamycin was incubated at 37 ℃ for 3 days. At the end of incubation, the glucose-treated LDL was dialyzed 1mM EDTA containing 67 mM phosphate buffer at 4 ℃. The dialyzed sample was reffered to G-LDL, LDL which was incubated without glucose and dialyzed according to the same procedure as described above was referred to U-LDL. Prior to use, both samples were dialyzed against 67 mM phosphate buffer to remove EDTA. Amounts of glucose incorporated into apoprotein B, which had been assayed by thiobarbituric acid method after hydrolysis of LDL by oxalic acid (6), were calculated to be approximately 10 mol/mol of apoprotein B, on the assumption of molecular weight of 500 K. This corresponds to modification of 2.8 % of lysine residues in apoprotein B.

Incubation conditions. The standard reaction mixture contained 50 μg of G-LDL (or U-LDL), 60 μM Fe^{3+}-1mM ADP, and 67 mM phosphate buffer at pH 7.4, in a total volume of 100 μl. Fe^{3+}-ADP complex (1:16.6 M/M) was prepared as described previously (9). Reaction was initiated by the addition of Fe^{3+}-ADP and continued during the time cited at 37 ℃ with a gentle agitation.

α-tocopherol and probucol, which were obtained from Eisai Company and Ohtsuka Pharmaceutical Co.Ltd., respectively, were dissolved in

ethanol before use. These were added to G-LDL so that ethanol concentration was 2 % in the reaction mixture.

Assays. Extent of lipid peroxidation of G-LDL or U-LDL was monitored by the formation of thiobarbituric acid reacting substance (TBARS) (10). Modification of G-LDL or U-LDL after exposure to Fe^{3+}-ADP was assessed by electrophoresis (see above). Cholesterol in G-LDL or U-LDL was estimated by cholesterol oxidase-phenol method (Wako Pure Chemical).

RESULTS

Contents of glycated LDL in G-LDL

G-LDL or U-LDL solution (270 μg protein/300 μl) was adjusted to pH 8.5 by 0.1 N NaOH and applied to a phenylboronate affinity column (PBA-10, Amicon, 1×10 cm) equilibrated with 100 mM phosphate buffer at pH 8.5. The column was developed first with 100 mM phosphate buffer and then with the same buffer which contained 100 mM sorbitol. The 0.5 ml-fractions were collected and LDL was estimated by cholesterol contents. As shown in Fig.1, glycated LDL which is retained on the column was 84 % in G-LDL, though U-LDL possessed no affinity to the phenylboronate.

Lipid peroxidation of glycated LDL by iron

It has been found that glycated polylysine-Fe^{3+}-complex as well as Cu^{2+}, induces lipid peroxidation of LDL (7) and of microsomal phospholipid

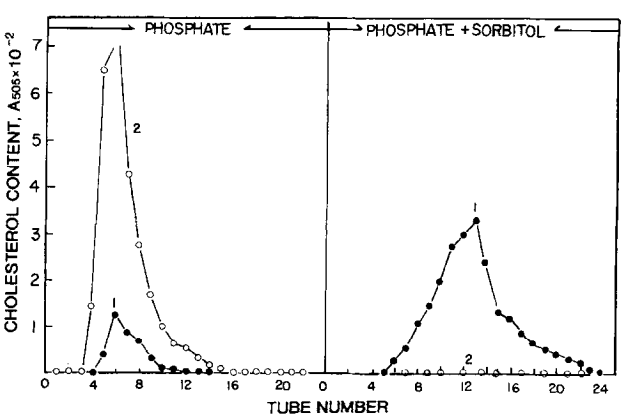

Fig.1. Phenylboronate affinity chromatography.
Column was developed first with 100 mM phosphate buffer at pH 8.5. The retained LDL on the column was eluted with 100mM sorbitol-containing 100 mM phosphate buffer at pH 8.5. (1) G-LDL (270 μg/300 μl), (2) U-LDL (270 μg/μl).

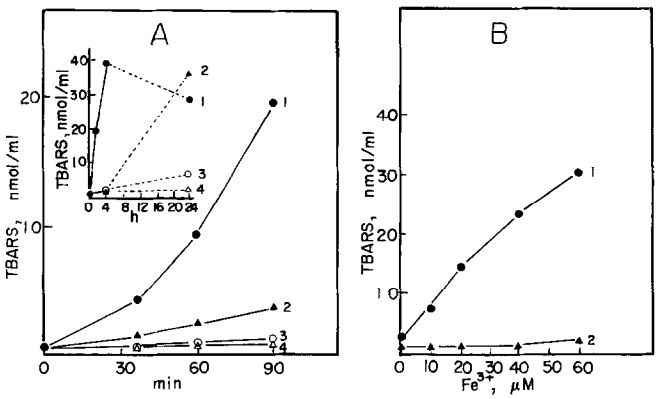

Fig.2. Lipid peroxidation of G-LDL and U-LDL induced by iron (A) and effect of concentrations of ferric ion (B).
(A) G-LDL or U-LDL (100 μg/200μl) was incubated with Fe^{3+}-ADP (60 μM) in 67 mM phosphate buffer (pH 7.4) at the indicated time, TBARS was assayed. (1) G-LDL + Fe^{3+}, (2) U-LDL + Fe^{3+}, (3) G-LDL, (4) U-LDL.
(B) G-LDL or U-LDL (100 μg/200 μl) was incubated with various concentrations of Fe^{3+}-ADP for 4 h, at 37℃. (1) G-LDL, (2) U-LDL.

liposomes (6). When G-LDL or U-LDL was incubated with or without Fe^{3+}-ADP, only the systems containing Fe^{3+}-ADP obviously showed lipid peroxidation which was monitored by the formation of TBARS. Time course of the lipid peroxidation are shown in Fig.2A and inset, in which iron-induced lipid peroxidation of G-LDL increased almost linearly up to 4 h after a short lag time and rather decreased at 24 h thereafter. Such a decrease of TBARS value during the long time incubation may be due to an expense of lipid peroxidation products (malondialdehyde and alkenals) for modification of apoprotein B. On the other hand, iron-induced lipid peroxidation of U-LDL did not significantly increased during 4 h-incubation, but obviously increased during additional 20 h. With a fixed concentration of G-LDL or U-LDL, the effects of iron concentrations during 4 h-incubation were examined. The lipid peroxidation of G-LDL increased in proportion to the iron concentration though no significant increase in TBARS was observed on U-LDL. These results are shown in Fig.2B.

Denaturation of apoprotein in G-LDL

To investigate denaturation of apoprotein moiety in G-LDL, the samples exposed to iron were subjected to SDS-polyacrylamide gel

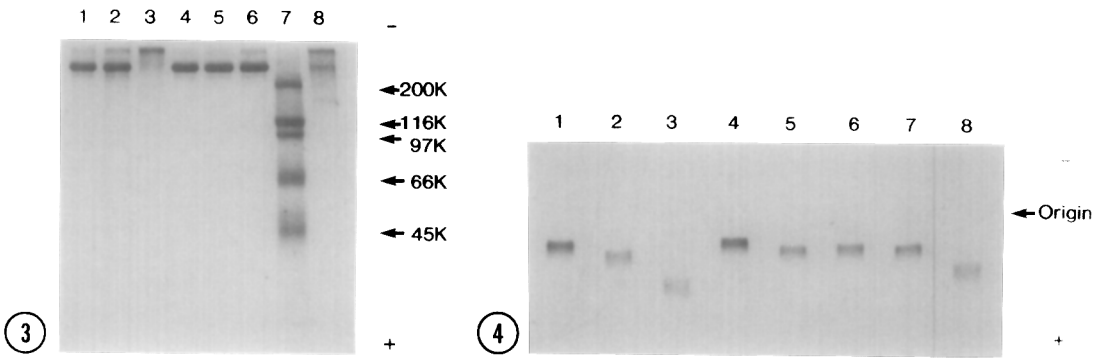

Fig.3. SDS-PAGE of G-LDL and U-LDL incubated with ferric ion.

The standard reaction mixture was used for iron-induced reaction. Native LDL (45 μg/ 100 μl) was also incubated with Cu^{2+} (5 μM) in 67 mM phosphate buffer. At the end of reaction, equal volume of the treatment buffer solution, which contains 0.125 M Tris-HCl at pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.003 % BPB and 2 mM PMSF was added to an aliquot (30 μl) of each reaction mixture and 10 μl of the treated sample was applied on the gel. The 10 % running gel and 5 % stacking gel were used. Marker (Biorad) contained myosin, B-galactosidase, phosphorylase B, bovine serum albumin and ovalbumin.
lane 1; 0 h-Fe^{3+}-exposed G-LDL, lane 2; 4 h-Fe^{3+}-exposed G-LDL (sample A), lane 3; 24 h-Fe^{3+}-exposed G-LDL (sample B), lane 4; 0 h-Fe^{3+}-exposed U-LDL lane 5; 4 h-Fe^{3+}-exposed U-LDL, lane 6; 24 h-Fe^{3+}-exposed U-LDL (sample D), lane 7; marker, lane 8; 4h-Cu^{2+}-exposed native LDL (sample C).

Fig.4. Electrophoretic mobilities of G-LDL or U-LDL incubated with or without ferric ion on agarose gel.

G-LDL or U-LDL was incubated under the same conditions as in Fig.3. At the end of reaction, an aliquot (1 μl) of the reaction mixture was run on the gel and stained by Fat Red 7B. lane 1; 0 h-Fe^{3+}-exposed G-LDL, lane 2; 4 h-Fe^{3+}-exposed G-LDL (sample A), lane 3; 24 h-Fe^{3+}-exposed G-LDL (sample B), lane 4; 0 h-Fe^{3+}-exposed U-LDL lane 5; 4 h-Fe^{3+}-exposed U-LDL, lane 6; 24 h-Fe^{3+}-exposed U-LDL (sample D), lane 7; 24 h-non treated G-LDL, lane 8; 4h-Cu^{2+}-exposed native LDL (sample C).

electrophoresis (Fig.3) and to agarose gel electrophoresis (Fig.4). G-LDL, which had been exposed to Fe^{3+}-ADP for 4 h (sample A), showed at least two bands on the SDS gel plate, i.e., one with the same molecular weight as that of untreated G-LDL (protein I) and the other with higher molecular weight (protein II). The protein II of G-LDL exposed to Fe^{3+}-ADP for 24 h (sample B) significantly increased with a great decrease of protein I. Such a great change in apoprotein moiety of LDL was also observed, when LDL was incubated with 5 μM Cu^{2+} for 4 h (sample C). However, only trace amount of protein II was detected in U-LDL exposed to Fe^{3+}-ADP for 24 h (Sample D). When samples A to D and others were run on the agarose gel and stained by the Fat Red 7B, only the samples B and C,

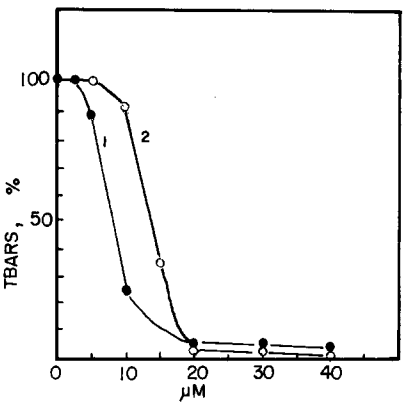

Fig.5. Effects of α-tocopherol and probucol on lipid peroxidation of G-LDL induced by ferric ion.
G-LDL (50 μg/100 μl) was preincubated with α-tocopherol (1) or probucol (2) at various concentrations for 15 min at 37 ℃, and then exposed to 60 μM Fe^{3+}-ADP for 4 h.

which contained protein II to greater extent, moved faster than untreated G-LDL or LDL to anode (Fig.4).

Effects of antioxidants on the lipid peroxidation of G-LDL induced by iron

When α-tocopherol or probucol was added to G-LDL and then exposed to 60 μM Fe^{3+}-ADP in the standard reaction mixture for 4 h, the lipid peroxidation was strikingly inhibited, i.e., 50 % inhibition was achieved by 7 μM α-tocopherol or by 13 μM probucol. Results were shown in Fig.5.

DISCUSSION

The present results clearly indicated that both apoprotein and lipids in G-LDL, but not in LDL, were denatured and oxidized in the presence of a naturally occurring transition metal, Fe^{3+}, during in vitro incubation. As suggested previously (6), Fe^{3+} could be coordinated with endiol group in Amadori compound derived from glycated peptide and could be converted to ferryl iron with a high redox potential. It seems likely, therefore, that G-LDL produces ferryl type complex to initiate lipid peroxidation, and the resulted lipid peroxidation products cause the modification of

apoprotein, identical to the case of the oxidative modification of LDL by glycated polylysine-Fe^{3+}-complex (7). Under the conditions in which both apoprotein and lipid in G-LDL could be oxidatively modified, α-tocopherol or probucol which was exogeneously added, possessed powerful inhibitory action on the lipid peroxidation, probably also on the denaturation of apoprotein.

Significantly high glycation of apoprotein of LDL in plasma from hyperglycemic diabetic subjects has been reported (11). Oxidatively modified LDL which can be phagocytized by macrophage accumulates in atherosclerotic lesions. Thus, glycated LDL, which could be coordinated with iron, may be a candidate for promoting atherosclerosis in diabetic patients. This is the first observation for contributing to the glycation to oxidative modification of LDL.

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