J. Food Sci. Nutr.
Vol. 1, No. 1, p. 93~98(1996)

Effects of Lipid Peroxidation of LDL and Lp(a)

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Abstract

Lipoprotein(a)[Lp(a)] is a macromolecular complex found in human plasma that combines structural elements composed of LDL and apo(a), and that is associated with premature coronary heart disease and stroke. In this study, various samples which consisted of normal and abnormal LDL and Lp(a) were selected for comparison. The above samples were incubated with copper in order to oxidize and to compare atheroma formation, *in vitro* and free radical formation of Lp(a) was decreased compared to purified LDL. And LDL from a 40 year old donor was higher in the free radical formation than that from a 20 years old donor. In order to investigate the macrophage foam cell formation, oxidized LDL or Lp(a) was incubated with human monocyte derived macrophage(HMDM). Oxidized samples enhanced on acceptability of foam cell formation by HMDM were compared to the control group. Also, structural changes of LDL and Lp(a) against oxidation times were found from HPLC mapping.

Key words: LDL, Lp(a), atherosclerosis, lipid oxidation, free radical, human monocyte derived macrophage(HMDM)

INTRODUCTION

Human low density lipoprotein(LDL) is a main carrier for cholesterol in the bloodstream, and it is well established that cholesterol deposits in the arteries stem primarily from the LDL and that increased levels of plasma LDL correlate with an increased risk of atherosclerosis(1,2).

LDL are removed from the circulation by both high affinity receptor-mediated and receptor-independent pathways, the liver being the major organ responsible for the LDL clearance(3). Apoprotein(apo) B-100 is the major protein constituent in LDL and is the ligand recognized by the LDL receptor. It is one of the largest monomeric proteins known, with a calculated molecular weight of 513 KD(4).

Lipoprotein(a)[Lp(a)] is a macromolecular complex found in human plasma that combines structural elements composed of LDL and apo(a), and is associated with premature coronary heart disease and stroke (5-7). It is assembled from the low density lipoprotein and a large hydrophilic glycoprotein called apolipoprotein (a) which is homologous to the protease zymogen plasminogen(8,9). Elevated plasma Lp(a) levels have been identified as a strong independent risk factor for premature development of atherosclerosis involving both coronary and cerebral arteries(4-9).

Macrophages have a scavenger receptor, by which they can recognize and ingest modified LDL(10,11). Subsequent research showed that scavenger receptors also bind oxidized LDL; a modification is more likely to occur *in vivo*(12-14).

In the course of lipid metabolism, some circulating LDL particles migrate across the endothelial border and enter the arterial subendothelial space. This itself is normal, but if there is an excess of LDL, a significant proportion of those LDL particles can undergo oxidative modification. Circulating LDL, modified or not, is swept up and reprocessed by the liver(11,12). Among the polyunsaturated fats, arachidonic acid is of particular interest. A key byproduct of arachidonic acid oxidation is malondialdehyde(MDA). MDA can directly alter the conformation of apo B, the predominant lipoprotein of LDL. LDL particles carrying MDA-modified apo B have a diminished affinity for the normal LDL receptor and an increased likelihood of being taken up by a macrophage(11,12,14). Unfortunately, macrophages do not downregulate their scavenger receptors after consuming their fill of cholesterol ; instead, they turn into giant, obese, cholesterolester-rich foam cells, the key ingredients of atheromas. Foam-cell growth through scavenger receptor uptake of oxi-

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dized LDL is one cornerstone of current models of atherogenesis, but there are other ways modified LDL can contribute as well(15-18).

Oxidized LDL particles have increased levels of lysolecithin, which both attracts circulating monocytes into the subendothelial space where they become macrophage and inhibits subendothelial macrophages from migrating elsewhere. Macrophages themselves can secrete potentially atherogenic substances; by inhibiting macrophage migration, oxidized LDL gives those substances a better chance of being secreted where they can do the most harm(12,16).

The purpose of this study was to determine effects of lipid oxidation on LDL-containing groups, such as LDL and Lp(a), and to compare the acceptability of foam cell formation by HMDM between normal and abnormal(oxidized) LDLs.

MATERIALS AND METHODS

Materials

Two male diabetic patients were recruited from the College of Medicine, Hallym University. Normal LDLs were collected from two healthy men, 20 and 40 years old, to compare the difference of LDL on age. 5-Iodoacetamide fluorescein(5-IAF) was obtained from Molecular Probes(Eugene, OR, USA). Fetal calf serum(FCS) was purchased from Cal Biochem(La Jolla, CA). EGTA, Dulbecco's modified Eagle's medium(DMEM), penicillin, streptomysine sulfate, and Ficoll-Histopaque were purchased from Sigma Chemical Company(St. Louis, MO, USA).

Isolation of LDL and Lp(a)

Plasma freshly obtained from a Lp(a)-positive donor was collected in 0.02% sodium azide, 1mM EDTA, and 10units/ml kallikreine inactivator, and immediately subjected to differential ultracentrifugation. The plasma was adjusted with solid KBr to d 1.05g/ml and centrifuged at 40,000rpm for 24hr, 4°C. The lipoproteins that floated were removed by pipetting and the infranatant was adjusted with solid KBr to d 1.08g/ml and recentrifuged. The d 1.050~1.080g/ml fraction was then dialyzed against 20mM Tris(pH 7.4), 1M NaCl, 1mM EDTA, 0.02% NaN_3, and were subjected to gel filtration chromatography on a Sephacryl-(S) 400 HR column(2.6×90cm) equilibrated with the same buffer at a flow rate of 12ml/hr. Fractions that eluted from the column were monitored by UV detector.

LDL were isolated from the d 1.025~1.055g/ml fraction. The purity of this fraction was tested by SDS-PAGE. Purified lipoprotein samples were stored at 4°C in 0.15M NaCl containing 0.54mM EDTA.

Electrophoretic techniques

SDS-PAGE was performed on 10% gels using an electrophoresis buffer consisting of 25mM Tris, 192 mM glycine and 0.1% SDS(pH 8.3). Samples were diluted in a sample buffer consisting of 10mM Tris(pH 8.8), 1mM EDTA, 50g/L SDS. They were incubated at 95°C for 10min. before addition of glycerol and tracking dye to aid application to the gels. Electrophoresis was performed at a constant 30mA for 5 to 6hr. The gels were fixed for 20min in 30% methanol, 20% acetic acid, 61% distilled-water and stained for 1hr in 1% Coomassie Blue R 250 in 10% acetic acid. Destaining was performed in 63% distilled-water, 7% acetic acid, and 30% methanol.

Oxidation of LDL and Lp(a)

The Lp(a) and LDL samples prepared by ultracentrifugation were dialyzed(to remove EDTA) against 0.01M phosphate-buffered saline(PBS)(pH 7.4) for 12 hours. The dialysis buffer was made oxygen free by vacuum degassing and was changed three times during dialysis(the dialysis bags were treated for 30min in boiling water before use).

The dialyzed sample containing 0.40mg lipoprotein was transferred to a 50ml tube and brought to a final volume of 1ml with 0.01M PBS. To initiate the oxidation, 16μl of $CuSO_4$(1mM) were added. The final copper concentration was 16μM in all cases(11,12). The kinetics of the reaction of the lipoproteins was followed by measuring the free radical formation at different incubation times.

Peptide mapping by HPLC

Structural changes of oxidized LDL containing groups were detected on a Waters HPLC system equipped with a UV detector. A Vydac C18 reverse phase column was equilibrated with a trifluoroacetic acid(TFA) buffer system at a flow rate of 1.0ml/min (buffer A: 0.1% TFA in water, buffer B: 0.08% TFA in 95% acetonitrile plus 5% water(v/v/v)). A linear gradient system

of buffer was increased from 0 to 70% at a rate of 1ml/min. The eluted fractions were monitored at 254nm.

Separation of human monocyte-derived macrophage

Individual cellular elements were separated by layering and centrifuging in a discontinuous density gradient formed by Hypaque(a mixture of sodium diatrizoate), Ficoll(a polysaccharide), and isotonic saline-diluted blood. After the centrifugation, mononuclear cells, platelets, and basophils are cleanly separated from the other element. Macrophages can be separated from the other mononuclear cells by a second separation step utilizing dextran.

5-IAF labelling of lipoproteins

5-IAF(5-Iodoacetamide Fluorescein) was dissolved in 0.10M ammonium bicarbonate(pH 8.0), and added to the LDL containing groups(15mg). The mixture was stirred in the dark for 24 hours at room temperature. To remove unbounded 5-IAF(free form), the mixture was applied to a Sephadex G-50 column(3×20cm) equilibrated with PBS(pH 7.4) at a flow rate of 1.5ml/min. The fractions containing 5-IAF-LDL complex were pooled and concentrated using a ultrafiltration kit(Spectrum, USA).

RESULTS AND DISCUSSION

Isolation of Lp(a) and LDL

Plasma was subjected to differential ultracentrifugation to obtain various fractions. The Lp(a) was found predominantly in the d 1.05~1.08 and much lesser amounts in the d<1.05 or the d>1.21 fraction. The d 1.05~1.08 fractions were used for further purification of Lp(a) by gel filtration chromatography. Lp(a) was eluted earlier than LDL because Lp(a) is consisted of LDL and apo(a)(19-21), larger molecular weight than LDL. It was difficult to separate LDL from Lp(a) by gel filtration chromatography because of their similar size(Fig. 1). Only those Lp(a) containing fractions(black parts) were pooled and used for further studies. The purification of LDL and Lp(a) were identified using a SDS-PAGE(10% polyacrylamide gel)(Fig. 2).

Measurement of free radical formation

The susceptibility of Cu^{+2} mediated oxidation of Lp

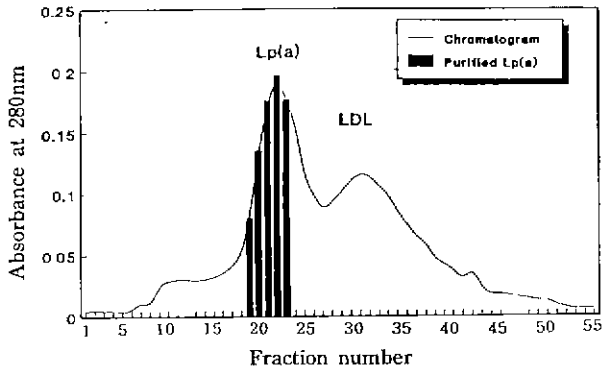

Fig. 1. Gel filtration chromatogram of Lp(a).
The Sephacryl 400 HR column(1.5×90cm) was equilibrated with 20mM Tris-saline buffer, pH 7.4 and the density fraction 1.05<d<1.08g/ml was chromatographed at flow rate of 12ml/h. The fractions were analysed for A_{280}. ■ ; purified Lp(a).

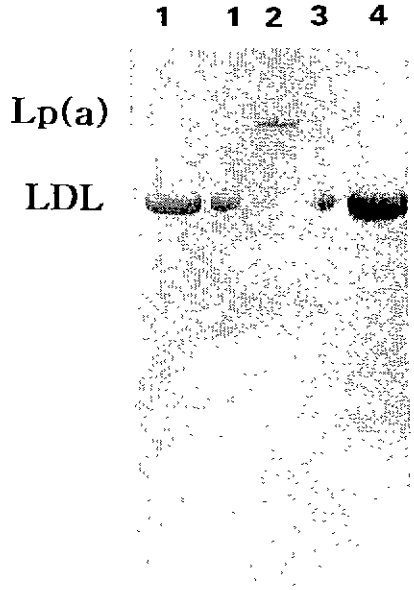

Fig. 2. SDS-PAGE of gel filtration chromatogram.
Protein loading was 20μg per fractions and gel stained with 1% coomassie Blue. lane 1: fraction number: 28-40, lane 2: fraction number 19-24, lane 3: fraction number 9-18, lane 4: purified LDL(standard)

(a) was studied and compared with that of LDL. The amount of free radical formed was measured during oxidation. Changes of the reaction pattern as determined in these experiments are shown in Fig. 3. The rate of free radical formation was considerably lower in the case of Lp(a) as compared to LDL. The decrease in the free radical amount at later times(after 60min) is most probably due to the degradation of hydroperoxy fatty

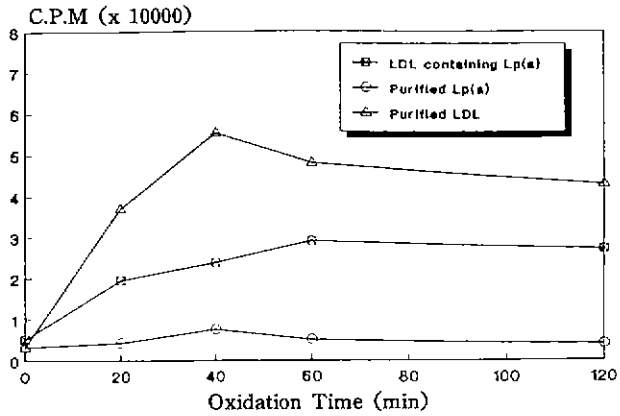

Fig. 3. Changes of free radical formation of LDL containing groups.

Table 1. Comparison of free radical formation of LDL between 20 and 40 year donors

Oxidation time (min)	40 years donor ($\times 40^4$ C.P.M)	20 years donor ($\times 10^4$ C.P.M)
0	0.32	0.32
20	3.70	0.89
40	5.54	1.10
60	4.81	1.22
120	4.30	1.40

acids to aldehyde degradation products(11,12). And it may lead further consequence to modification of the apoprotein and recognition by the macrophage scavenger receptor(14,15).

Based on the oxidation rate, purified Lp(a) was somewhat more resistant against oxidative modifications than purified LDL(Fig. 3). Also, true peroxide formation in LDL from a 20 year old donor was retarded in comparison to LDL from a 40 year-old donor(Table 1). From this result, it is proved that functional deterioration of macromoecules, such as LDL, is progressed with advancing age(22). It is regarded that the altered function is viewed as a molecular basis of aging(2,22,23).

Peptide mapping by HPLC

After the oxidation of LDL and Lp(a) at various incubation time, HPLC was used to compare the difference of LDL containing groups. for the studies of structural changes. Fig. 4 exhibited that HPLC chromatogram of LDL containing groups, such as normal LDL, diabetic LDL and Lp(a). It showed the different pattern on the retention times between 20 and 30min. Lp(a) which contained LDL, was resisted on fragmentation of apolipoprotein by oxidation compared to

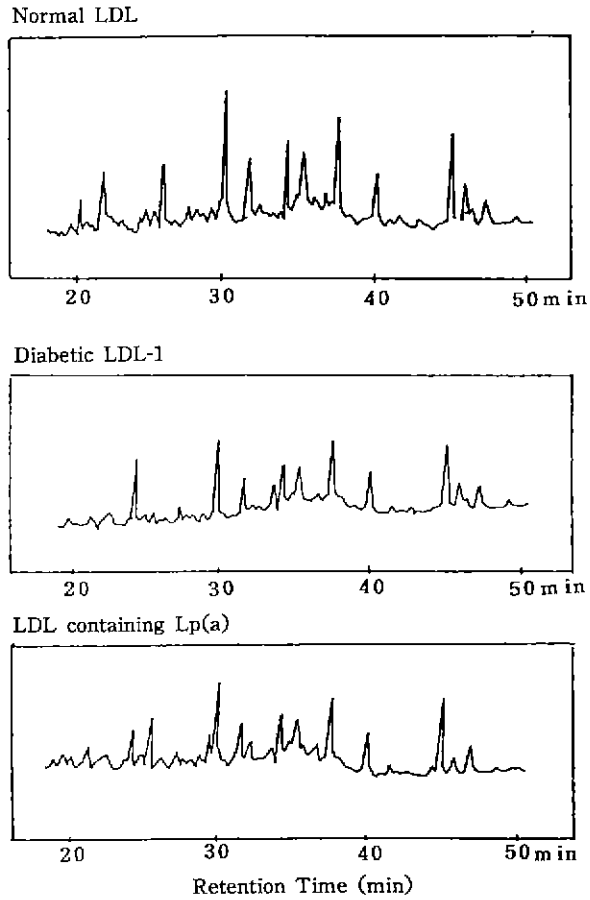

Fig. 4. HPLC Chromatogram of LDL containing groups.

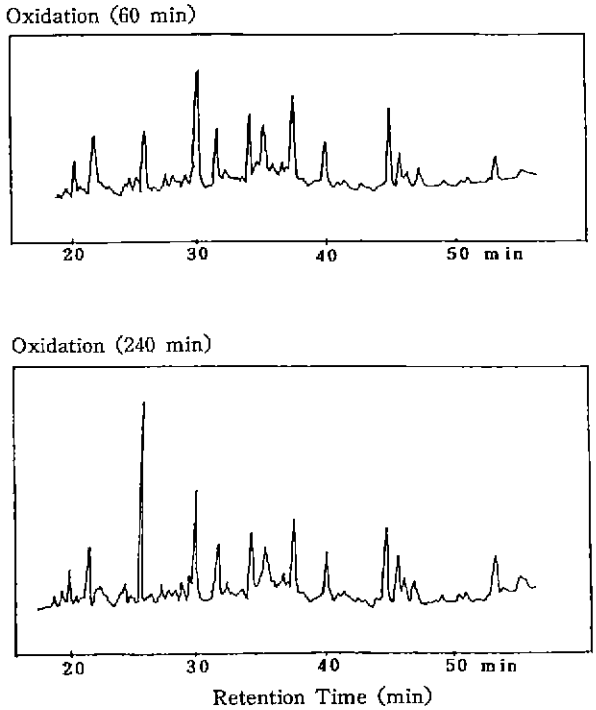

Fig. 5. HPLC Chromatogram of normal LDL. (donor' 40 years. 254nm, 40°C)

normal LDL. Also, it was found that diabetic LDL showed a different apoprotein distribution compared to normal LDL. It is hypothesized that nonenzymatic reactions of glucose, a reducing sugar, with amino group of apoproteins results in a series of events which alter structure and function of protein(15).

Fig. 5 showed the structural changes of apoprotein against oxidation time. In the case of normal LDL, as the time of oxidation increased, the fragmentation of apolipoprotein increased.

Many other changes are associated with the oxidative degradation of the LDL, such as, an extensive fragmentation of the apo B to smaller peptides(18,19). It seems very likely that these phenomena, together with the covalent binding of aldehydes, lead to a complete structural rearrangement of the protein creating new epitopes which do not bind to the B/E receptor but to the scavenger receptor(4,19).

Degradation studies of oxidized LDL by HMDM

LDL was labeled with 5-IAF for the degradation studies. Fig. 6 showed absorption spectra of IAF-LDL containing groups and its maximum spectra of 5-IAF was 493nm. For the oxidation, labeled lipoproteins were incubated with 16μM Cu^{+2}. Oxidized lipoproteins were incubated for 12 hours at 37°C with human monocyte-derived macrophage(HMDM) for the experiment of degradation pattern. Degradation of oxidized LDL was measured according to the decreased amount

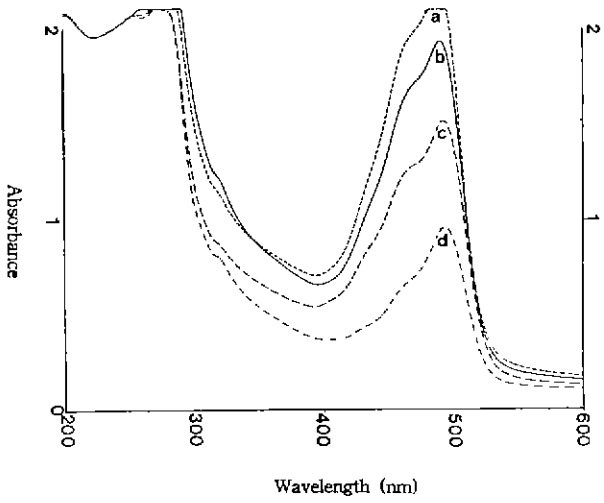

Fig. 6. Absorption spectra of IAF-LDL containing groups.
a: diabetic-I, b: diabetic-II
c: 40 years donor, d: 20 years donor

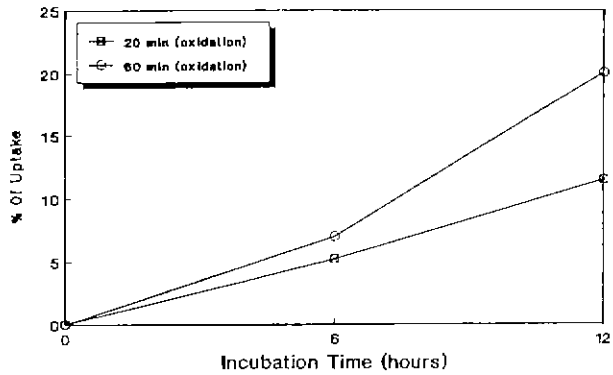

Fig. 7. Uptake of oxidized LDL by HMDM according to incubation time.
(40 years donor)

of fluoresence. The amount of fluorescence was quantified using a SFM-25 spectrofluorimeter.

Fig. 7 showed that the increase of oxidation time led the enhanced interaction of LDL and HMDM. The binding of modified LDL to the scavenger receptor seems to afford certain clusters of negative charges at the apo B, and an artificial increase of the negative surface charges by chemical modifications. It results in an increased recognition by the scavenger receptor and an unlimited uptake of cholesterol(12,14).

Macrophages also means scavenger receptor which mediates the endocytosis of several forms of modified LDL(22,24). This receptor is not under the control of intracellular cholesterol. In this culture, the uptake of modified LDL by the scavenger receptor lead to the accumulation of cholesterol which is then stored in the form of lipid droplets(14,25).

From the results of oxidative effects, Lp(a) exhibited resistance on lipid oxidation and protein degradation compared with purified normal LDL. LDL showed the highest free radical formation followed by LDL containing Lp(a) and pure Lp(a). And LDL from a 40 year old donor was higher in the free radical formation than that from 20 year old donor. Abnormal LDLs from diabetic patients were much higher in the free radical formation than normal LDL.

In order to investigate the macrophage foam cell formation, oxidized LDL containing groups were incubated with human monocyte derived macrophage. Oxidized samples were enhanced on acceptibility of foam cell formation by HMDM compared to control groups. 60min(oxidation time) increased the normal LDL uptake by HMDM as compared to 20min. Abnormal LDL from diabetic patients showed the decreased LDL

uptaked to control groups.

It was concluded that abnormal high glucose levels of LDL from diabetic patients showed a quite different patterns in oxidation and HPLC mapping compared to normal LDL. From these studies, oxidized LDL enhanced the possibility of atherosclerosis and, the high carbohydrate levels of Lp(a) retarded oxidative effects by copper. But, this result dose not mean that Lp(a) act as anti-atherosclerotic factor.

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(Received May 26, 1996)