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# Functional Properties of Modified Low Density Lipoprotein and Degradation of Modified LDL by Human Monocyte-Macrophages

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## Abstract

**Human plasma low density lipoprotein (LDL) is the main carrier for cholesterol, and recent studies suggest the normal LDL can be readily oxidized by free radical and not interact with LDL receptor. Lipoprotein particles are consisted of lipid and protein, and fatty acids of lipoproteins are prone to oxidation. LDL particles readily undergo oxidative modification by copper. From the results, oxidized LDL altered its biological properties. A marked increase in the electrophoretic mobility of LDL on agarose gel indicated that negative surface charge of the LDL particles was increased. Also, the results from the HPLC showed that oxidized LDL was degraded into several polypeptides nonenzymatically. Degradation tests which measured the amount of 5-IAF labelled oxidized LDL were carried out by monocyte and hepatocyte cell culture. Hepatocyte cell culture of modified LDL did not show consistent pattern. However, binding rate of modified LDL with HMDM (human monocyte derived macrophage) was enhanced with oxidation, but was retarded by addition of antioxidants (hyaluronic acid, vitamin A, vitamin E). Also comparisons of oxidized-LDL, acetyl-LDL and MDA-LDL showed significant differences in the chemical properties and binding affinity to HMDM. Thus, modification of normal LDL altered its biological properties.**

**Key words :** oxidized LDL (ox-LDL), acetyl LDL, MDA-LDL, rat hepatocyte, human monocyte derived macrophage (HMDM)

## INTRODUCTION

Oxidative modification of LDL has been shown to be associated with increased atherogenicity which includes cytotoxicity to arterial wall cells stimulation of homostatic and thrombotic processes, and secretion of cytokines and growth factors from cells of arterial wall. An important feature of oxidized LDL (ox-LDL) is its enhanced uptake by macrophages, which results in cellular cholesterol accumulation and foam cell formation, a characteristic of the early atherosclerotic lesion. LDL oxidation can be achieved *in vitro* by lipoprotein incubation with cells of the arterial wall including endothelial cells, smoth muscle cells, and macrophages (1-4).

The first demonstrated effect of LDL oxidation was its increased absorption of cultured macrophages. LDL incubated with endothelial cells was degraded by macrophages three to five times more rapidly than lipoprotein that is incubated in the absence of cells. The uptake of cell modifed LDL was predominantly via a high-affinitied, saturable pathway, acetylated LDL competed with cell-modified LDL but the competition was partial. A study with acetylated LDL and endothelial cell-modified LDL shows that there are at least two scavenger receptors for these modified particles and that cell-modified LDL and acetylated LDL are not identical ligands, the intracellular processing of degradation of each is different (5,6).

Various lines of research provide strong but not conclusive evidence that LDL may become oxidized *in vivo* and that oxidized LDL is the species involved in the formation of early atherosclerotic lesions. Early atherosclerotic lesion is characterized by massive accumulation of cell filled with lipid droplets consisting of cholesterol and cholesterol esters. Because of their

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foamy appearance such cells are called foam cells (3-5).

In culture, macrophages take up native LDL only slowly, and even if incubated over long periods with high LDL concentrations, they do not accumulate cholesterol esters and transform to lipid-laden cells. This receptor is not under the control of intercellular cholesterol. The majority of these cells are macrophages and derived from circulating monocytes-macrophages (8). It is currently believed that post secretory modifications of LDL may render the lipoprotein more atherogenic. Recent studies have suggested that oxidatively modified LDL may represent one such modified form of LDL (6,7). Copper-mediated oxidation of LDL had properties similar to endothelial cell-mediated oxidation of LDL that different in the several ways compare to native LDL (6,7).

Previous study was undertaken to investigate the structual and functional changes of oxidized LDL and compare properties of mildly and extensively oxidized LDL (2). In this paper, degradation pattern of modified LDL by rat hepatocyte cell and human monocyte derived macrophage (HMDM), and the inhibitory effects of antioxidants against LDL oxidation, were studied.

## MATERIALS AND METHODS

### Materials

Dulbecco's modified Eagle's medium (DMEM), EGTA, streptomycine sulfate, penicillin, 2-thiobarbituric acid (TBA), trypsin inhibitor, Ficoll-Histop aqueous solution, aprotinin, and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). 5-Iodoacetamide fluorescein (5-IAF) was obtained from Molecular probes (Eugene, OR, U.S.A.). Fetal calf serum (FCS) was purchased from Cal Biochem (La Jolla, CA, U.S.A.). Barbital sodium was purchased from Junsei Chemical Co. (Tokyo, Japan). Trichloroacetic acid (TCA) was obtained from Janssen Chimical (Geel, Belgium). Acetonitrile and other solvents for HPLC were purchased from J. T. Becker (Philipsberg, NJ, U.S.A.).

### Preparation of LDL

Aprotinin (0.05units/ml), EDTA (0.05%, w/v) and sodium azide (0.05%, w/v) were added to plasma from healthy donors to prevent for coagulation and further hydrolysis (9). The LDL, $1.025<d<1.055$g/ml, was isolated by sequential ultracentrifugation using KBr gradient at 40,000rpm for 15hrs at 4°C with a Beckman 50.2 Ti rotor, followed by one more runs for 24hrs under the same conditions. The protein was analyzed by electrophoresis on 1% SDS/5~14% gradient polyacrylamide slab gel and was shown to contain only one protein band at the position of apo B-100. LDL was dialyzed against EDTA-free PBS (phosphate buffered saline) (7).

### Modification of LDL

#### (A) Preparation of oxidized-LDL

IAF-LDL was incubated with cupric sulfate (5$\mu$M) at a concentration of 400$\mu$g/ml protein in 1ml PBS in the presence and absence of antioxidants (40$\mu$M ascorbic acid, 40$\mu$M $\alpha$-tocopherol, and 10nM hyaluronic acid) at 37°C for each times. The extent of lipid peroxidation was measured as thiobarturic acid reactive substances (TBARS) and expressed as MDA equivalents (6,8).

Briefly, 1.0ml of 25% trichloroacetic acid (TCA) was added to 0.5ml samples, followed by 1.0ml of 1% TBA. The samples were vortexed and incubated at 95°C for 50min, then they were centrifuged (1000× g) for 15min. Supernatant TBARS were detected by UV-spectrophotometer at 532nm. The freshly diluted 1,1,3,3-tetramethoxypropan (TMP) were used as standards.

#### (B) Preparation of acetyl-LDL

To ice-cold LDL, 2mg/ml in PBS-EDTA, an equal volume of saturated sodium acetate solution was added with continuous stirring in an ice-water bath. Acetic anhydride in multiple 1.5~2.0$\mu$l amounts was added over a period of 1hr. for the total mass of anhydride, 1, 1.3 and 1.5 times the mass of the lipoprotein protein. And stirred in ice-water bath for 30min and dialysed extensively against PBS containing 0.01% EDTA.

For analysis of reactive amino groups by trinitro-

benzene sulfonic acid (TNBS), 2.0ml TNBS solution (0.1%, w/v) was added to each aliquots. Contents were mixed and incubated in a covered water bath at 50° C. After 60min incubations the reaction was stopped by added 4.0ml of 0.1N HCl. The samples were then allowed to cool for 30min, and measured at 340 nm (10).

$$\text{Degree of reactive amino groups} = \frac{\text{O.D. of unmodified LDL} - \text{O.D. of modified LDL}}{\text{O.D. of unmodified LDL}} \times 100$$

### (C) Preparation of MDA-LDL

1nM of TMP was treated with 1ml of 4N HCl for 30min at 50° C to generate free malondialdehyde (MDA). The solution was then neutralized with 4N NaOH to pH 7.0 and made to 2.5ml with PBS. LDL (5mg) was treated with 100, 150, 200 and 250$\mu$l of MDA solution at 4° C (added in 20 equal aliquots at 5min intervals with stirring) and the stirring was continued to 2hr. The MDA-LDL was dialysed extensively against PBS-containing 0.01% EDTA (11).

### Agarose gel electrophoresis

To determine an increased electrophoretic mobility of modified-LDLs, agarose gel electrophoresis was carried out on 0.5% agarose gel in 0.05M barbital buffer, pH 8.6 (2).

### HPLC

Structural changes of modified LDL was tested on a Water's HPLC system equipped with a variable wavelength detector (10). The column temperature was set for 50° C. For chromatogram, a Vydac $C_{18}$ reverse phase column (1.0 × 25cm) was used with trifluoroacetic acid (TFA) buffer system at a flow rate of 1.5ml/min, buffer A : 0.1% TFA in water (v/v), buffer B : 0.08% TFA in 95% acetonitrile plus 5% water (v/v/v). A linear gradient of buffer was increased from 0 to 60% at a rate of 1%/min. The eluted peptide fractions were monitored at 220nm.

### Cell culture

Rats hepatocyte cell culture was carried out as follows (12). Rats who had free access to food and water, were anesthetized. After cannulation of the vena porta, perfusion is started with Hank's buffer, at 37° C pH 7.4. Preperfusion, 10min with flow rate, 14ml/min was done with $Ca^{2+}$-free Hank's buffer containing 1.9g/L of EGTA. After the initial 10min, perfusion was continued for 20min with Hank's buffer containing collagenase (0.05%), trypsin inhibitor (0. 005%) and 1mM $Ca^{2+}$. The liver was removed and transferred to a petri dish, and chopped in normal Hank's buffer and filtered twice through nylon gauze. The filtration was transferred to 50ml of plastic cap centrifuge-tube with 25ml of normal Hank's buffer and centrifuged two times at 700rpm for 5min. The pelletes were suspended with 20ml of DME medium containing 10% FCS. Human monocyte-derived macrophages (HM-DM) were isolated from human whole blood with 10IU of heparin per ml of blood. The blood was layered onto Ficoll-Histopaque (1 : 1, v/v) and centrifuged at 1,800 × g for 25min at 10° C. Cells at the interface were removed and washed twice in Hank's buffer. They were then resuspended in the DME medium supplemented with 10% FCS. The viability of HM DM and hepatocyte cell was determined by tryphan blue dye and the exclusion exceeded 89% and 80, respectively, in all experiments.

### Labelling of LDL by 5-iodoacetamide fluorescein (5-IAF)

5-IAF was dissolved in 0.10M amonium bicarbonate, pH 8.0, and added to the LDL (100-fold molar excess of 5-IAF). The mixture was stirred in the dark for 24hrs at room temperature. To eliminate excess reagent , the sample was applied to a 3 × 20cm Sephadex G-50 column and eluted with PBS, pH 7.4. The fractions containing fluorescent material eluted in the void volume were pooled and lyophilized (10).

### Degradation study of modified IAF-LDL

The dagradation amounts of modified LDL by cultured cells were measured using SFM-25 spectrofluorometer with 1ml cuvette at 25° C with excitation wavelength at 493nm and emission wavelength at 550nm (13).

## RESULTS AND DISCUSSION

### Chemical modification of LDL

To compare with oxidized LDL, LDLs (MDA and acetyl-LDL) were modified mildly and extensively with chemical treatments (Fig. 1). Acetyl-LDL was prepared by the addition of acetic anhydrides (1, 1.3 and 1.5 times mass of the lipoprotein protein) to LDL. Modified ratio of lysine were 31.2, 51.3 and 75.3%, respectively. Also, MDA-LDL was prepared by TMP (100, 150, 200, and 250μl) with LDL. Modified ratio of lysine residues were 20, 34.4, 59.8 and 83.7%, respectively. Superko (16) reported that once 16% of the lysine residues on the apo B protein have been modified by MDA, the new configuration became recognizable by the macrophage scavenger receptor.

### Agarose gel electrophoresis

Relative electrophoretic mobility (REM) from 0.5% agarose gel electrophoresis of oxidized LDL and chemically modified LDL are shown on Table 1. REM of modified LDL showed the increased mobility compared to native LDL. Chemically and oxidatively modified-LDLs might be blocked of positive charge of lysine residues. Fong (8) reported that oxidation of LDL by copper ion resulted in a consistent decrease in histidine (32%), lysine (15%), and proline (10%) residues as well as a smaller decrease in methionine (6%), while the amounts of aspartic acid actually increased (6%). Therefore, modified LDLs could show more increased electrophoretic mobilities than those for native LDL. But in the presence of ascorbic acid, electrophoretic mobility of oxidized LDL decreased remarkably, although it showed more increased mobility than native LDL. From the above results, amino acid composition of apo B was changed during LDL oxidation, and negative charge of LDL particles was increased. It was proved that ascorbic acid have notable effects of antioxidant to reduce LDL oxidation.

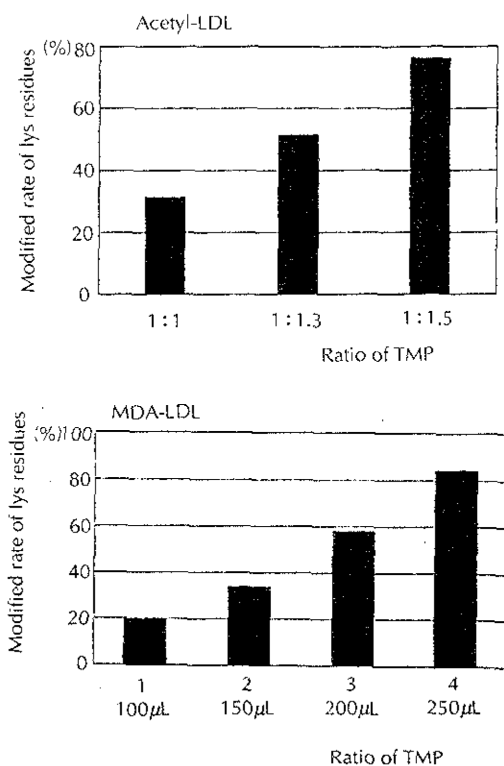


**Fig. 1. Chemical modification of LDL.**

### Studies of structural changes of oxidized LDL using HPLC

Structural changes of LDL after lipid peroxidation were measured on HPLC system with a reverse phased column (Fig. 2). HPLC chromatograms were compared with REM from agarose gel electrophoresis for structural changes of oxidized LDL. Oxidized LDL caused the severe structual changes compared with native LDL. Wavelength 220nm is very specific to detect peptide rather than polypeptide. Oxidized LDL at 220nm showed several peaks (between 10 and 20min) which mean polypeptide of LDL were frag-

**Table 1. Relative electrophoretic mobility (REM) of modified LDL[1)]**

| Conditions of modified LDL | Relative electrophoretic mobility |
|---|---|
| Native LDL | 1.00 |
| Ox-LDL (4hr) | 2.29 |
| Ox-LDL (6hr) | 2.31 |
| Ox-LDL (12hr) | 2.35 |
| Ox-LDL (24hr) | 2.75 |
| + ascorbate (40μM) | 1.16 |
| + tocopherol (40μM) | 1.91 |
| MDA-LDL | 2.79 |
| Acetyl-LDL | 2.62 |

[1)]Electrophoretic mobility is expressed as migration relative to native LDL

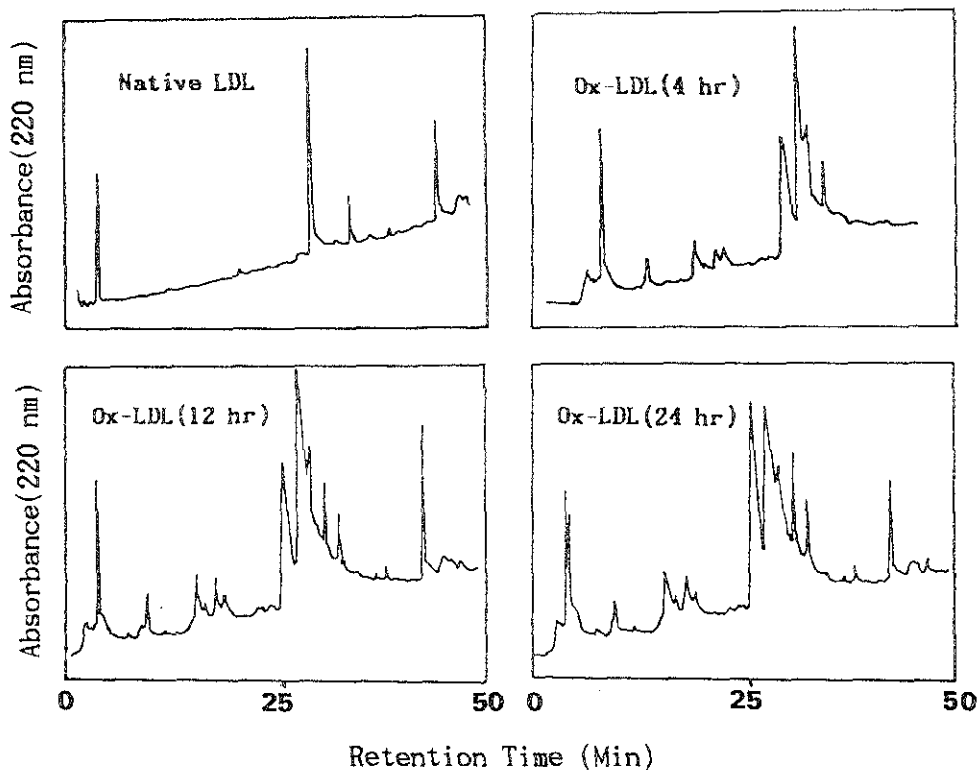


Fig. 2. **HPLC chromatogram of ox-LDL at 220nm.**

mented into smaller peptides. From the results by agarose gel electrophoresis and HPLC, native LDL was degraded non-enzymatically into several polypeptides and amino acid composition of apo B-100 was changed. Therefore, native LDL including its polypeptide component, apo B-100, lost its nascent form during the LDL oxidation. Also, Parthasarathy *et al.* (14) reported that during the oxidative modification of LDL, the apo B-100 is degraded non-enzymatically into several large polypeptides.

### Degradation studies of oxidized LDL by monocyte and hepatocyte cell

For the degradation studies, fluorescent probe, 5-iodoacetamide fluorescein (5-IAF) was used for labelling of LDL. Absorption spectra of IAF-LDL was shown in Fig. 3, and maximum spectra of 5-IAF was 493nm. To test the degradation of oxidized LDL, rat liver, major site of LDL receptor production, was used to determine the interaction (Fig. 4). Oxidized LDL

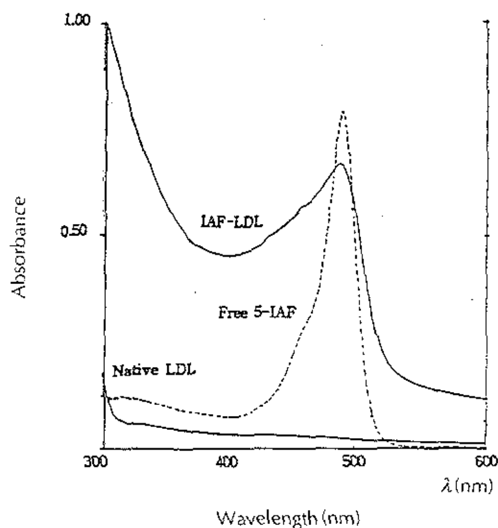


Fig. 3. **Absorption spectra of IAF-LDL.**

was incubated with collagenase treated rat hepatocyte cells. From the results, degradation amounts of modified LDLs by cultured rat hepatocyte cells did not

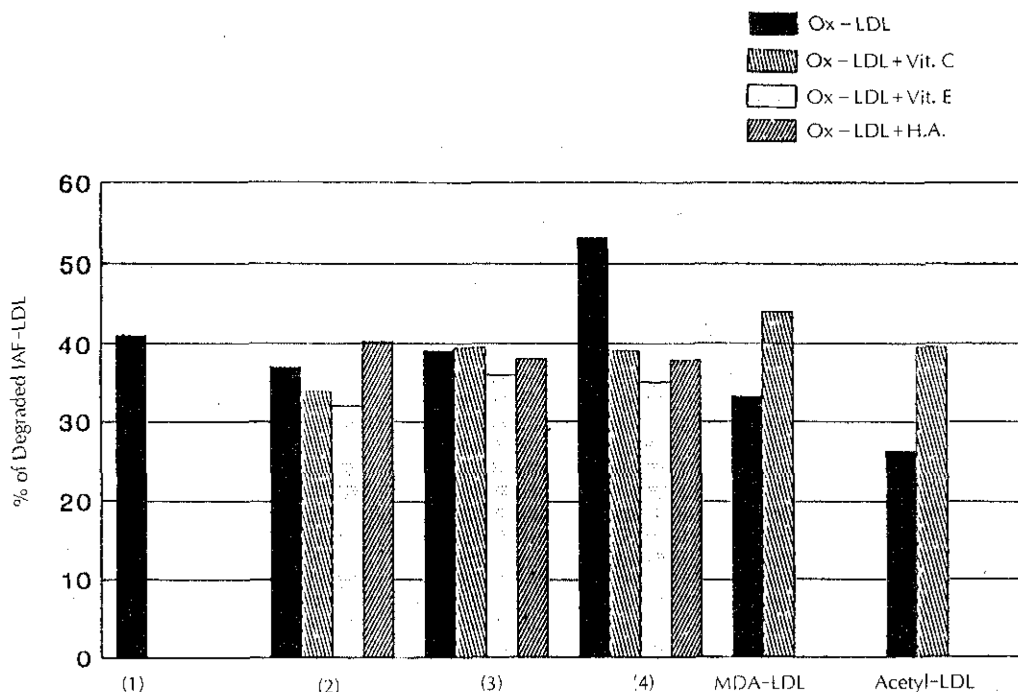


**Fig. 4. Degradation pattern of modified LDL by rat hepatocyte cells[1].**
(1) Oxidation time : 0hr (2) Oxidation time : 4hr (3) Oxidation time : 6hr (4) Oxidation time : 12hr
[1]Each oxidized LDL was labelled with 5-IAF and incubated with rat hepatocyte cell for 24hrs at $CO_2$ incubator for the degradation test. The higher the percent of degraded IAF-LDL, the higher the binding capacity of LDL and hepatocyte cell.

show consistent pattern because rat liver cell contains not only LDL-receptor but kuffer and endothelial cell. Heinecke *et al.* (9) studied binding capacity of kuffer and endothelial cell. After a separation of rat river cells into parenchymal, endothelial, and kuffer cells, and 10min after injection of ox-LDL, binding capacity is 6.8-fold higher than for acetyl-LDL, leading to kuffer cells as the main liver site for ox-LDL uptake. Also, liver contains LDL receptor.

One of the major biological effects of ox-LDL is its increased rate of uptake and degradation via the scavenger receptor of macrophages. Hence, in the presence study, human monocyte derived from macrophage was used for the study of the degradation of ox-LDL, in addition to the effect of antioxidants on this biological effect by assaying the degradation of ox-LDL by HMDM.

As shown in Fig. 5, oxidized LDL was more obviously degraded by HMDM compared to native LDL. During the 12 hr incubation time with HMDM, degradation rate of ox-LDL was increased linearly. On

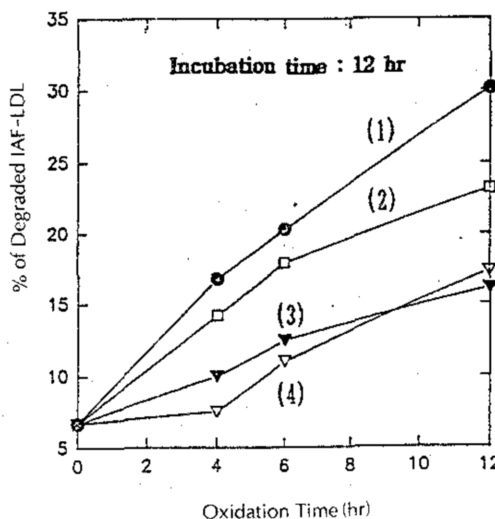


**Fig. 5. Degradation of ox-LDL by human monocyte derived macrophage(HMDM).**
(1) Ox-LDL
(2) Ox-LDL + H.A.
(3) Ox-LDL + Vit. C
(4) Ox-LDL + Vit. E

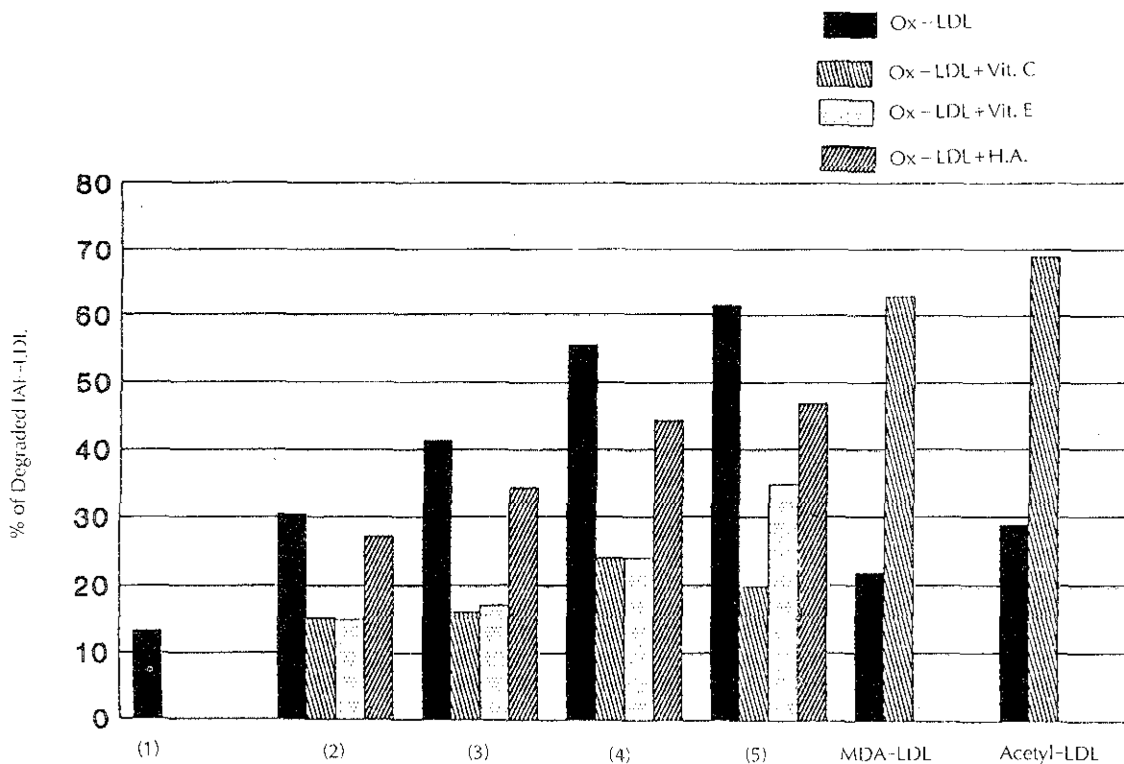


**Fig. 6. Degradation pattern of modified LDL by human monocyte derived macrophage (HMDM)[1].**
(1) Oxidation time : 0hr (2) Oxidation time : 4hr (3) Oxidation time : 6hr (4) Oxidation time : 12hr
(5) Oxidation time : 24hr
[1]Each oxidized LDL (labelled with 5-IAF) was incubated with HMDM for 24hrs at $CO_2$ incubator for the degradation test. The binding of LDL and HMDM is enhanced as the percent of degraded IAF-LDL increased.

the other hand, binding rate of ox-LDL by HMDM was inhibited. In the presence of antioxidants (ascorbate, $\alpha$-tocopherol and hyaluronic acid), hyaluronic acid decreased the uptake rate of ox-LDL by HMDM, it means that hyaluronic acid is less effective than ascorbate and $\alpha$-tocopherol.

At a 24hr incubation with HMDM, binding curves were revealed to be hyperbolic pattern (Fig. 6). $\alpha$-Tocopherol was less effective antioxidant on LDL oxidation. Binding rate of ox-LDL was increased about two-times of that in the presence of ascorbate, although lower than that in the absence of ascorbate. From the results, $\alpha$-tocopherol had an inhibitory effect on the oxidative modification of LDL only for 12hrs, as evidenced by a increased TBARS, the amounts of free radicals (2,15) and electrophoretic mobility on agarose gel (Table 1). However ascorbate severely inhibited the oxidative modification degree of LDL after 24hrs incubation. Hyaluronic acid (0.005%) had a much less antioxidant effect on LDL oxidation. All antioxidants, ascorbate (40$\mu$M), $\alpha$-tocopherol (40$\mu$M) and hyaluronic acid (0.005%, w/v), showed the inhibition of the oxidative modification of LDL.

To compare the degradation rate of chemically modified LDL with oxidized LDL by HMDM, chemically modified LDLs, acetyl-LDL and MDA-LDL were employed (Fig. 6). At a 24hr incubation with HMDM, mildly chemically modified acetyl-LDL and MDA-LDL (modification rates of Lys residues were 31.2 and 19.8%, respectively) showed the low degradation rate, i.e., below 30% which was similar to mildly oxidized LDL (4hr oxidation), but extensively chemically modified acetyl-LDL and MDA-LDL were degraded severely, similar to 24hr oxidized LDL by HMDM. Therefore, extensive modified LDLs (12 and 24 hr) changed its biological properties similar to extensively chemically modified LDLs.

In conclusions, LDL particles undergo readily oxi-

dative modification by copper. Therefore, oxidatively modified LDL altered its structural and functional properties in addition to the interaction with macrophage scavenger receptor. Factors promoting lipoprotein oxidation are due to aging, and reduced antioxidative defence systems in the body. Oxidized LDL has another biological effect that is potentially important in the development of atherosclerosis (16). It interacts with platelets, which promotes their aggregation, a phenomenon that native LDL stimulates to a lesser extent.

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# 변형된 형태의 저밀도 지질단백질(Modified LDL)의 특성 및 대식세포와의 결합 연구

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## 요 약

인간의 혈장 저밀도 지단백(LDL)은 관상동맥경화 발병의 주요 요인이다. 그러나, 최근의 연구에 의하면, 정상적인 LDL은 산소 자유라디칼에 의해 쉽게 산화되며, 결과 LDL 수용체와 결합하지 못한다고 밝히고 있다. 따라서 이 변형된 형태의 LDL은 macrophage scavenger receptor에 의해 인식되어 거품세포(foam cell)를 형성하여, 동맥혈관이 좁아지는 역할을 수행한다. 지질과 단백질로 구성되어 있는 LDL의 산화에는 실제 작용성을 가진 단백질 보다는 지질이 절대적인 작용을 한다. 정상적인 LDL을, 산화 LDL(oxidized LDL), acetyl-LDL 및 MDA-LDL의 세종류의 변형된 LDL(modified LDL)로 만들었으며, 이의 정도를, TNBS에 의한 lysine의 modification rate과 agarose gel 전기영동상에서의 이동도를 측정하였다. 이때, 항산화제인 비타민 C, 비타민 E 또는 hyaluronic acid가 첨가되면 LDL의 산화가 억제되는 효과를 확인하였다. 또한 HPLC를 이용하여 산화된 LDL이 효소의 작용없이 여러개의 덩어리로 분해되는 것을 확인할 수 있었다. 5-LAF로 표지된 변형 LDL이 사람의 monocyte에서 유래된 macrophage(HMDM)와 쥐의 간세포에 의해 얼마나 분해되는가를 실험하였다. 그 결과, 배양된 간세포는 변화된 형태의 LDL을 분해하는데 일정한 형식을 나타내지 못했고, 모든 종류의 LDL을 거의 비슷하게 분해하였다. 그러나 HMDM에 의한 변형된 LDL의 분해는 saturation kinetics를 보여주었다. 따라서, 정상적인 LDL이 modified LDL로 전환된다는 것은, 생리적인 특성이 크게 변화된다는 것을 의미한다. 또한 oxidized-LDL과 acetyl-LDL, MDA-LDL을 비교하였을 때, 상당히 유사한 형태를 보여 주었다. 이와같은 결과로 부터, 정상적인 LDL에 비교하였을 때, 상당히 유사한 형태를 보여 주었다. 이와 같은 결과로부터, 정상적인 LDL에 비해 노화나 오염된 공기, 또는 약물 등 외적인 요인에 의해 발생되는 산화된 형태의 LDL은 동맥경화의 위험인자로 볼 수 있다.