主　論　文

Lipid peroxide and transition metals are required for the toxicity of oxidized low density lipoprotein to cultured endothelial cells

(酸化自己変性低比重リポ蛋白の培養血管内皮細胞に及ぼす障害作用に関する研究
— 特に過酸化脂質ならびに遷移金属の関与について —)

Masafumi Kuzuya, Michitaka Naito, Chiaki Funaki, Toshio Hayashi, Kanichi Asai and Fumio Kuzuya

葛谷雅文　内藤通孝　舟木千明　林登志雄　浅井幹一　葛谷文男

Department of Geriatrics, Nagoya University School of Medicine, Tsuruma-cho, Showa-ku, Nagoya 466, Japan

Address for correspondence: Dr. Masafumi Kuzuya, Department of Geriatrics, Nagoya University School of Medicine, Tsuruma-cho, Showa-ku, Nagoya 466, Japan.

Key words.

Oxidized low density lipoprotein; endothelial cell; atherosclerosis; lipid peroxidation; cytotoxicity; copper.

Abbreviations: LDL, low density lipoprotein; Ox-LDL, oxidized LDL; TBARS, thiobarbituric acid-reacting substances; DME, Dulbecco's modified Eagle's medium; MEM, Eagle's minimum essential medium; EDTA, ethylenediaminetetraacetic acid; LDH, lactate dehydrogenase; LPO, lipid hydroperoxides; MDA, malondialdehyde.

Summary

The toxicity of oxidized low density lipoprotein (Ox-LDL) to cultured vascular endothelial cells was investigated. The modification of low density lipoprotein (LDL) by copper led to the production of thiobarbituric acid-reactive substances (TBARS) and lipid hydroperoxides (LPO). TBARS was distributed not only in lipoprotein but also in the aqueous phase, whereas LPO was observed only in the lipoprotein particle. During the incubation of LDL with copper, the copper bound to lipoprotein and formed a complex. The toxicity of products resulting from the oxidation of LDL to endothelial cells was recognized in Ox-LDL particles, not in the aqueous phase. Following dialysis of Ox-LDL against EDTA, copper which had bound to the Ox-LDL particle was released and the toxicity of Ox-LDL disappeared. The addition of copper to the dialyzed Ox-LDL restored the cytotoxicity. To a lesser extent this effect was also observed with the addition of iron. A study of the time course of LDL oxidation showed that the toxicity of Ox-LDL depends upon the level of LPO, not upon the content of TBARS, the extent of negative charge or the protein adduct of aldehydes. These results demonstrate that transition metal is required for Ox-LDL toxicity and that the toxic moiety of the products resulting from LDL oxidation is LPO associated with the Ox-LDL particle.

Introduction

Recent studies suggest that the oxidative modification of low density lipoprotein (LDL) may play an important role in the initiation and progression of atherosclerosis [1,2]. LDL can be modified by incubating it with endothelial cells [3], smooth muscle cells [4,5] or monocytes/macrophages [6,7] in the presence of trace amounts of transition metals. The oxidative modification of LDL is variously associated with lipid peroxidation [8], an increase in net negative charge [4], hydrolysis of phospholipid [8] and fragmentation of apoprotein B [8,9]. Recently it has become apparent that this biological modification is mediated via a free radical-induced peroxidation of LDL which can be mimicked by such transition metals as copper or iron in the absence of cells [8,10]. Oxidative modification of LDL increases its uptake by macrophages through the scavenger receptor [11,12] and stimulates monocyte chemotaxis [13,14]. Oxidized LDL (Ox-LDL) also exhibits toxicity to various cells including cultured vascular endothelial cells [15-17]. Although the endothelial damage caused by Ox-LDL would be relevant to atherogenesis, the detailed mechanism of cell injury induced by Ox-LDL is unknown. Previous studies suggest that the toxin of Ox-LDL resides in the lipid phase [15] and that the susceptibility to Ox-LDL depends upon the phase of the cell cycle [18] and on the level of intracellular glutathione [19]. It is not clear, however, how Ox-LDL induces injury to the target cell, and what toxic substances may be contained in Ox-LDL.

This study focuses on these questions using cultured bovine aortic endothelial cell as the target cell. The evidence presented indicates that the toxic moiety of Ox-LDL is the lipid hydroperoxide (LPO) as-

sociated with the Ox-LDL particle, and that such a transition metal as copper is required for the induction of LPO-dependent Ox-LDL toxicity to the target cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DME) and phenol red free Eagle's minimum essential medium (MEM) were purchased from the Nissui Pharmaceutical Co. (Tokyo, Japan). EDTA was obtained from Katayama Chemicals (Osaka, Japan) and 2-thiobarbituric acid from Wako Chemicals (Osaka, Japan). Bovine calf serum was obtained from Cell Culture Laboratories (Cleveland, USA). Flasks and 16 mm 24-well plates were obtained from Corning Glassworks (Corning, USA).

Cell Culture

Endothelial cells were established in culture from the thoracic aorta of the fetal calf as previously described [20]. They were maintained in DME supplemented with 10% (v/v) calf serum, penicillin, streptomycin and amphotericin in a humidified atmosphere containing 5% CO_2 and 95% air. Cells at passage 9-13 were used for all experiments.

Preparation of Oxidized Low Density Lipoprotein

LDL (density 1.019 to 1.063) was isolated from normal human plasma by ultracentrifugation as previously described [21]. The LDL preparation was dialyzed at 4℃ for 48 h against two changes of at least 100 volumes of 0.15 M NaCl (pH 7.4). For the preparation of Ox-LDL, LDL was diluted

with saline to the indicated concentration and incubated with 20 μM $CuSO_4$ at 37℃ for specified intervals. In some experiments, aliquots were then dialyzed against at least 100 volumes of 0.15 M NaCl (pH 7.4) for 24 h at 4℃ or dialyzed against 0.15 M NaCl with 0.1 mM EDTA (pH 7.4) for 24 h followed by further dialysis against 0.15 M NaCl (pH 7.4) for 24 h at 4℃ to exclude EDTA.

Gel Filtration of Oxidized LDL

One ml of sample was diluted with 1 ml of saline and loaded onto a column of Sephadex G-25M (PD-10, Pharmacia, Uppsala, Sweden) and eluted with saline. Fractions of 1 ml volume were collected and assayed for protein, TBARS, LPO, copper and cytotoxicity. The content of protein, TBARS, or LPO was recovered more than 90% following gel filtration.

Assays for Products of Lipid Peroxidation

The thiobarbituric acid-reacting substances (TBARS) in 100 μl of the sample were measured fluorometrically as previously described [19]. Malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxypropane was used as a standard. The lipid hydroperoxides (LPO) in 100 μl of the sample were determined using the Determiner LPO kit (Kyowa Medex Co., Tokyo, Japan) according to the methods of Ohishi et al. [22-24]. Cumene hydroperoxide was used as a standard. The fluorescence intensity of Ox-LDL was measured on a spectrofluorometer (Hitachi F-3010) with excitation at 360 nm, emission at 430 nm and excitation at 400 nm, emission at 470 nm as protein adducts of reactive aldehydes [2].

Assessment of Injury to Endothelial Cells

Cellular injury was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the cells into the medium. Endothelial cells at confluence in 24-well plates (approximately 5.3×10^5 cells/well) were rinsed with MEM twice and incubated in a total volume of 0.4 ml of MEM containing 30% (v/v) Ox-LDL for 16 h at 37℃ except where noted. MEM used in the present study lacks transition metals including copper. In some experiments, $CuSO_4$ in stock solution (100 μM in MEM) was added to the test medium at the concentrations indicated in the text. Following incubation, LDH activity in the medium was determined by spectrophotometric analysis of NADH oxidation (Shimadzu spectrophotometer UV-160) [25]. Each LDH activity was compared with that released from cells following the addition of Triton X-100 for 0.1% (v/v) final concentration (% total LDH release).

Other Methods

For the determination of polyunsaturated fatty acids in Ox-LDL, lipids were extracted from the samples according to Folich et al. [26]. Lipids were saponified with methanolic potassium hydroxide followed by methylation with 14% (w/v) BF_3-methanol reagent [27]. Fatty acid methyl esters were analyzed on capillary gas chromatography (Hewlett-Packard, 5890A) equipped with a flame ionization detector, using a 25 m × 0.2 mm Carbowax 20 M column (Hewlett-Packard). Protein was assayed by the method of Lowry [28] using bovine albumin as the standard. Agarose gel electrophoresis of Ox-LDL was carried out as described by Nobel [29]. The concentration of copper was determined by atomic absorption spectrophotometry [30] using a Hitachi Model 180-60. Results are the means of duplicate determinations unless otherwise noted. Statistical

significance was determined by Student's t test. Probability level was $P<0.001$.

Results

Localization of Toxic Substance

After incubating LDL with 20 μM $CuSO_4$ for 24 h, 1 ml of the sample was diluted with 1 ml saline and applied to the Sephadex G-25M column. Fig. 1B shows the elution patterns of protein, TBARS and LPO. Two peaks of TBARS were found; the first was observed in fractions containing Ox-LDL protein, and the other, larger peak was seen in fractions not containing protein, indicating that TBARS is not only present in lipoprotein but also in the aqueous phase. The LPO and protein elution patterns coincided; no peak in LPO was observed that was not associated with Ox-LDL protein, indicating that in contrast to TBARS, LPO was located in lipoprotein, not the aqueous phase.

To evaluate the cytotoxicity of each fraction to cultured endothelial cells, the confluent cells in 24-well plates were exposed to the test medium consisting of MEM plus each fraction (40%, v/v) for 16 h at 37℃. Fig. 1A shows that the extent of the toxicity of each fraction to the cells was correlated with the content of protein and LPO of each fraction. The fractions containing free TBARS did not exhibit toxicity suggesting that the toxic substance is associated with the Ox-LDL particles, and that the presence of TBARS in the aqueous phase is nontoxic to endothelial cells.

To evaluate the localization of copper, separate gel filtration was

carried out to determine the concentration of copper in each fraction. Fig. 2 shows the elution patterns of copper and protein. Copper was found both in lipoprotein and in the aqueous phase, suggesting that copper exists not only in its free form but also bound to the Ox-LDL particle.

Effect of Dialysis on Ox-LDL Toxicity

After incubating LDL with 20 μM $CuSO_4$ for 24 h, the sample was dialyzed against 0.15 M NaCl containing 0.1 mM EDTA at 4℃ for 24 h followed by a further dialysis against 0.15 M NaCl for 24 h to remove EDTA. One ml of the sample was then diluted with 1 ml saline and applied to the column. Compared to Fig. 1B, Fig. 3C shows that the TBARS peak not associated with the Ox-LDL protein disappeared following dialysis, and that the peaks of TBARS and LPO associated with the Ox-LDL protein remained at the same fractions as before dialysis. Measurement of the concentration of copper in each fraction demonstrated an absence of copper from all fractions following dialysis against EDTA (Fig. 2).

Evaluation of cytotoxicity showed that the toxicity of those fractions which had exhibited a cytotoxic effect before dialysis disappeared completely after dialysis against EDTA (Fig. 3A). To examine whether the disappearance of this toxicity would be referable to the removal of copper by dialysis, a low concentration of $CuSO_4$ (2 μM) was added to the test medium. As shown in Fig. 3B, the effect of the fractions containing Ox-LDL on the toxicity to endothelial cells was restored to a pattern similar to that preceding dialysis.

After incubating LDL for 24 h with 20 μM $CuSO_4$, dialysis of Ox-LDL against 0.15 M NaCl without EDTA for 24 h demonstrated that copper remained in the lipoprotein particle but disappeared from the aqueous

phase (Fig. 2). The toxicity of the fractions containing Ox-LDL remained to the same extent as that of the nondialyzed sample (data not shown).

Requirement of Copper for Ox-LDL Toxicity

To further confirm that the copper is required for the toxicity of Ox-LDL to endothelial cells, we compared the cytotoxicity of Ox-LDL before and after dialysis (Fig. 4). Before dialysis Ox-LDL was toxic to the cells. Ox-LDL dialyzed against NaCl without EDTA was observed to have a toxic effect to the same extent as Ox-LDL without dialysis. However, no toxic effect was observed with Ox-LDL which had been dialyzed against EDTA followed by further dialysis against NaCl, at least over the periods of time studied (16 h). The addition of various concentrations of $CuSO_4$ to the test medium containing Ox-LDL dialyzed against EDTA led to a dose-related increase in the release of LDH. $CuSO_4$ at the concentrations used in this study had no effect on cell viability.

Effect of Duration of LDL Oxidation on Toxicity

To examine the effects of varying the level of oxidation of LDL on the toxicity to endothelial cells, LDL was incubated with 20 μM $CuSO_4$ at 37℃ for various intervals (0 h - 48 h). Samples were then dialyzed against 0.1 mM EDTA for 24 h and further dialyzed against 0.15 M NaCl for 24 h. During incubation with $CuSO_4$, Ox-LDL showed an increased electrophoretic mobility on agarose gel electrophoresis (Fig. 5), indicating an increased net negative charge during incubation. The kinetics of the various products in Ox-LDL resulting from lipid peroxidation, and the cytotoxicity of Ox-LDL are shown in Fig. 6. The TBARS content of Ox-LDL increased with an increasing length of incubation with $CuSO_4$ up to 48 h

(Fig.6A), while the extent of LPO was diphasic. LPO increased up to 16 h and then decreased at 24 h and 48 h (Fig.6A). The fluorescence intensities of Ox-LDL measured with excitation at 360 nm, emission at 430 nm and excitation at 400 nm, emission at 470 nm were also monitored as the formation of protein adducts of reactive aldehydes. The formation of these fluorophors increased during periods of incubation up to 48 h (Fig.6B).

Again in the absence of $CuSO_4$, there was no release of LDH from cultured cells with Ox-LDL at any level of oxidation. In the presence of 2μM $CuSO_4$, the extent of the toxicity of Ox-LDL was diphasic; there was an increased release of LDH up to 16 h, and with incubation periods longer than 24 h, the extent of LDH release decreased (Fig.6C), correlated closely with the level of LPO (Fig.6A). These results suggest that the toxic substance in Ox-LDL is LPO, not TBARS or protein adducts of aldehydes. Table 1 shows the amount of unsaturated fatty acids in Ox-LDL prepared by incubation with $CuSO_4$ for 0 h, 24 h and 48 h. Increasing the period of exposure to $CuSO_4$ led to a decrease in the content of polyunsaturated fatty acids in lipoprotein.

Discussion

Although the toxicity of Ox-LDL to a variety of cells is well documented [15-19], the detailed mechanism of cell injury and the identity of the toxic substance are poorly understood. It has been demonstrated that the toxic moiety of Ox-LDL resides in the lipid phase [15] and that the susceptibility to Ox-LDL depends upon the phase of cell cycle [18]. The

receptor-mediated uptake of Ox-LDL appears not to be required for cell injury, since Ox-LDL is also toxic to the cells which lack a scavenger receptor [15-17]. We recently found that the susceptibility of endothelial cells to Ox-LDL is dependent upon the intracellular glutathione level [19], and that the lipid peroxidation of the cellular membrane is involved in the cellular injury inflicted by Ox-LDL [31].

In this study we showed that the oxidative modification of LDL mediated by copper led to the production of TBARS in both lipoprotein and in the aqueous phase, consistent with the findings of other investigators [8,32]. LPO was present only in lipoprotein, not in the aqueous phase, indicating that TBARS in the aqueous phase is not derived from the breakdown of peroxides during the acid-heating stage of the TBA assay. TBARS in the aqueous phase is likely to consist of free MDA released from LDL fatty acids during the peroxidation process, as suggested by Esterbauer et al. [33] that most MDA formed during oxidation of LDL does not remain associated with LDL. Our observation that the toxic substance resides in the Ox-LDL particle, not in TBARS in the aqueous phase, agrees with the finding of Morel et al.[34].

We found that copper can bind to Ox-LDL and form a complex. Copper ions may bind to amino acids in lipoprotein during incubation with LDL, since copper ions readily ligate to the amino groups of protein [35]. Copper was released from the copper-Ox-LDL complex during dialysis against EDTA, suggesting that copper binds loosely to Ox-LDL and that the copper moiety can easily be released from the complex by a chelating agent such as EDTA. It is possible that the formation of the copper-lipoprotein complex may contribute not only to a change in conformation of lipoprotein but also lead to changes in its biological properties.

Although the free non-bound copper in the aqueous phase was removed during the dialysis of Ox-LDL against a dialysate without EDTA, the copper associated with the Ox-LDL particle, and the toxicity of Ox-LDL, both remained. In contrast the toxicity of Ox-LDL disappeared by removing the copper associated with the Ox-LDL particle during dialysis against EDTA. Furthermore, the addition of copper to the test medium containing Ox-LDL which had been dialyzed against EDTA restored this toxicity, indicating that copper is involved in the toxicity of Ox-LDL. Replacing copper with iron ($FeCl_3$, $FeSO_4$) in the test medium containing Ox-LDL dialyzed by EDTA also led to LDH release, although the effect of iron was weaker than copper (data not shown).

In the study of the time course of LDL peroxidation, we observed that TBARS content, electrophoretic mobility and protein adducts of aldehydes increased during the incubation of LDL with copper. It is well known that various aldehydes are formed from polyunsaturated fatty acids during the oxidation of LDL [33]. Of those aldehydes, 4-hydroxynonenal is a candidate for a toxic moiety in Ox-LDL, since this aldehyde has been reported toxic to certain cells [36-39]. A lipophilic aldehyde, 4-hydroxynonenal remains associated with the Ox-LDL particle, generating fluorophor (excitation 360 nm / emission 430 nm) in Ox-LDL protein [2,33]. MDA also reacts with lipoprotein, leading to a fluorescence maximum at excitation 400 nm / emission 470 nm [2]. However, it is unlikely that such aldehydes are responsible for the toxicity of Ox-LDL to endothelial cells, since in our results, the time-dependent increase in fluorescence of Ox-LDL was not consistent with the observed diphasic response of toxicity of Ox-LDL. The incubation of LDL with copper induced an initial increase in the LPO content of Ox-LDL then subsequently

decreased. This finding agrees with the recent report of Esterbauer et al.[40]. The decrease in LPO is explained by our observation that the long-term incubation of LDL with copper led to a depletion of polyunsaturated fatty acids resulting from the ultimate destruction of polyunsaturated fatty acids side-chains by lipid peroxidation [41]. In the absence of copper no toxic effect was detected with Ox-LDL. In contrast, in the presence of copper the toxic effect of Ox-LDL appeared and was strictly dependent on the level of LPO, not on the TBARS content, extent of negative charge or the fluorescence intensity of Ox-LDL. These results suggest that the toxicity of Ox-LDL is not dependent upon the extent of the oxidation of LDL, and that the most likely candidate for the toxic substance in Ox-LDL is LPO.

We demonstrated that LPO and such transition metals as copper or iron are required for the induction of Ox-LDL cytotoxicity. Pure LPO is fairly stable at physiological temperature. However, in the presence of transition metals including iron and copper, LPO is readily decomposed by a redox mechanism that generates such toxic products as free radicals and reactive aldehydes [42,43]. Therefore, the requirement of transition metal for LPO-dependent cytotoxicity implies that the toxic products generated from LPO during its decomposition would contribute to the lethal injury of cultured endothelial cells. If significant levels of hydroperoxides pre-exist in the Ox-LDL exposed to prooxidizing conditions, decomposition of these hydroperoxides would play a major role in the initiation of lipid peroxidation in the cell membrane leading to cell death.

References

1 Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) N. Engl. J. Med. 320, 915-924.

2 Jurgens, G., Hoff, H.F., Chisolm, G.M. and Esterbauer, H. (1987) Chem. Phys. Lipids 45, 315-336.

3 Henriksen, T., Mahoney, E.M. and Steinberg, D. (1981) Proc. Natl. Acad. Sci. USA 78, 6499-6503.

4 Henriksen, T., Mahoney, E.M. and Steinberg, D. (1983) Arteriosclerosis 3, 149-159.

5 Heinecke, J.W., Rosen, H. and Chait, A. (1984) J. Clin. Invest. 74, 1890-1894.

6 Cathcart, M.K., Morel, D.W. and Chisolm, G.M. (1985) J. Leukocyte Biol. 38, 341-350.

7 Parthasarathy, S., Printz, D.J., Boyd,D., Joy, L. and Steinberg, D. (1986) Arteriosclerosis 6, 505-510.

8 Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. USA 81, 3883-3887.

9 Fong, L.G., Parthasarathy, S., Witztum, J.L. and Steinberg, D. (1987) J. Lipid Res. 28, 1466-1477.

10 Heinecke, J.W., Baker, L., Rosen, H. and Chait, A. (1986) J. Clin. Invest. 77, 757-761.

11 Sparrow, C.P., Parthasarathy, S. and Steinberg, D. (1989) J. Biol. Chem. 264, 2599-2604.

12 Arai, H., Kita, T., Yokode, M., Narumiya, S. and Kawai, C. (1989) Biochem. Biophys. Res. Commun. 159, 1375-1382.

13 Quinn, M.T., Parthasarathy, S., Fong, L.G. and Steinberg, D. (1987)

Proc. Natl. Acad. Sci. USA 84, 2995-2998.

14 Quinn, M.T., Parthasarathy, S. and Steinberg, D. (1988) Proc. Natl. Acad. Sci. USA 85, 2805-2809.

15 Hessler, J.S., Morel, D.W., Lewis, L.J. and Chisolm, G.M. (1983) Arteriosclerosis 3, 215-222.

16 Evensen, S.A., Galdal, K.S. and Nilsen, E. (1983) Atherosclerosis 49, 23-30.

17 Morel, D.W., Hessler, J.R. and Chisolm, G.M. (1983) J. Lipid Res. 24, 1070-1076.

18 Kosugi, K., Morel, D.W., DiCorleto, P.E. and Chisolm, G.M. (1987) J. Cell. Physiol. 130, 311-320.

19 Kuzuya, M., Naito, M., Funaki, C., Hayashi, T., Asai, K. and Kuzuya, F. (1989) Biochem. Biophys. Res. Commun. 163, 1466-1472.

20 Ryan, U.S., Mortara, M. and Whitaker, C. (1980) Tissue & Cell. 12, 619-635.

21 Hatch, F.T. and Lees, R.S. (1968) Adv. Lipid Res. 6, 1-68.

22 Ohishi, N., Ohkawa, H., Miike, A., Tatano, T. and Yagi, K. (1985) Biochem. Int. 10, 205-211.

23 Tateichi, T., Yoshimine, N. and Kuzuya, F. (1987) Exp. Geront. 22, 103-111.

24 Yagi, K., Kiuchi, K., Saito, A., Kayahara, N., Tatano, T. and Ohishi, N. (1986) Biochem. Int. 12, 367-371.

25 Bergmeyer, H.U., Bernt, E. and Hess, B. (1965) Methods of Enzymatic Analysis, pp.736-741, Academic Press, New York.

26 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.

27 Metcalfe, L.D. and Schmitz, A.A. (1961) Anal. Chem. 33, 363-364.

28 Lowry, O.H., Rosebrough, N. J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

29 Nobel, R.P. (1968) J. Lipid Res. 9, 693-700.

30 Ichida, T. and Nobuoka, M. (1969) Clin, Chim. Acta 24, 299-303.

31 Kuzuya, M., Yamada, K., Hayashi, T., Funaki, C., Naito, M., Asai, K. and Kuzuya, F. (1990) Biochem. Int. (in press).

32 Yokode, M., Kita, T., Kikawa, Y., Ogorochi, T., Narumiya, S. and Kawai, C. (1988) J. Clin. Invest. 81, 720-729.

33 Esterbauer, H., Jurgens, G., Quehenberger, O. and Koller, E. (1987) J. Lipid Res. 28, 495-509.

34 Morel, D.W., DiCorleto, P.E. and Chisolm, G.M. (1984) Arteriosclerosis 4, 375-364.

35 Lau, S.J. and Sarkar, B. (1981) Biochem. J. 199, 649-656.

36 Benedetti, A., Comporti, H. and Esterbauer, H. (1980) Biochim. Biophys. Acta 620, 281-296.

37 Krokan, H., Grafstrom, R.C., Sundqvist, K., Esterbauer, H. and Harris, C.C. (1985) Carcinogenesis 6, 1755-1759.

38 Kaneko, T., Honda, S., Nakano, S. and Matsuo, M. (1987) Chem. Biol. Interact. 63, 127-137.

39 Kaneko, T., Kaji, K. and Matsuo, M. (1988) Chem. Biol. Interact. 67, 295-304.

40 Esterbauer, H., Rotheneder, M.D., Waeg, G., Striegl, G. and Jurgens G. (1990) Chem. Res. Toxicol. 3, 77-92.

41 Halliwell, B. and Gutteridge, J.M.C. (1989) Free Radicals in Biology and Medicine, pp.218-234, Clarendon Press, Oxford.

42 Girotti, A.W. (1985) J. Free Radical Biol. Med. 1, 87-95.

43 Halliwell, B. and Gutteridge, J.M.C. (1984) Biochem. J. 219, 1-14.

Fig. 1. Gel filtration of oxidized LDL. LDL (1.5 mg protein/ml) was incubated with 20 μM $CuSO_4$ for 24 h at 37℃, then diluted with saline (1:1, v/v). Two ml of the sample was loaded onto Sephadex G-25M column and eluted with saline. Fractions of 1 ml were collected and assayed for cytotoxicity (upper panel, A), protein(●), TBARS (△) and lipid hydroperoxides (LPO, ○) (lower panel, B). To assess cytotoxicity, endothelial cells at confluence in 24-wells were incubated in MEM containing each fraction (40%, v/v) at 37℃ for 16 h. LDH activity in the medium was then determined. Results are expressed as described in Materials and Methods. Values in A represent the means of duplicate wells and in B represent the means of duplicate determinations that differed by no more than 5% and 9%, respectively.

Fig. 2. Elution pattern of copper. Samples were diluted with saline (1:1, v/v) and 2 ml of aliquot was applied to Sephadex G-25M column and eluted with saline. Fractions of 1 ml were collected and assayed for protein and copper. The samples were prepared as follows; LDL (1.1 mg protein/ml) was incubated with 20 μM $CuSO_4$ for 24 h at 37℃ (○), then dialyzed against either 0.15 M NaCl for 24 h (△) or 0.15 M NaCl containing 0.1 mM EDTA for 24 h followed by further dialysis against 0.15 M NaCl for 24 h (□). The elution pattern of protein (●) was obtained from the sample which was prepared by incubation of LDL with 20 μM $CuSO_4$ for 24 h at 37℃. Other samples demonstrated the same elution pattern of protein (results not shown). Values represent the means of duplicate determinations that differed by no more than 10%.

Fig. 3. Gel filtration of oxidized LDL following dialysis against EDTA. LDL (1.5 mg protein/ml) was incubated with 20 μM $CuSO_4$ for 24 h at 37℃ and dialyzed against 0.15 M NaCl containing 0.1 mM EDTA for 24 h followed by further dialysis against 0.15 M NaCl for 24 h. The sample was then diluted with saline and applied to the column. The fraction (1 ml) eluted with saline was assayed as described in Fig. 1. In the assay for cytotoxicity, endothelial cells at confluence in 24-wells were incubated in MEM containing each fraction (40%, v/v) in the absence (upper panel, A) or presence (inset of upper panel, B) of 2 μM $CuSO_4$ at 37℃ for 16 h. Values in A and B represent the means of duplicate wells and in C represent the means of duplicate determinations that differed by no more than 5% and 9%, respectively.

Fig. 4. Effects of dialysis and copper on toxicity of Ox-LDL. LDH release was determined in the test medium after incubating endothelial cells in 24-wells with test medium for 16 h. The test medium consisted of MEM containing Ox-LDL (30%, v/v) prepared by one of the following methods: LDL (1.6 mg protein/ml) was incubated with 20 μM $CuSO_4$ for 24 h at 37℃ (without dialysis), then dialyzed against either 0.15 M NaCl for 24 h (dialysis against NaCl) or 0.15 M NaCl containing 0.1 mM EDTA for 24 h followed by further dialysis against 0.15 M NaCl for 24 h. Test medium containing varying concentrations of $CuSO_4$ was also assayed. Results are expressed in Materials and Methods. Values represent the means ± standard deviation of triplicate wells. *$P<0.001$ vs. Cu 0 μM.

Fig. 5. Mobility on agarose gel electrophoresis of variously oxidized LDL. LDL (1.5 mg protein/ml) was incubated with 20 μM $CuSO_4$ at 37℃ for 0 h (A), 8 h (B), 16 h (C), 24 h (D) and 48 h (E). Each sample was dialyzed against 0.15 M NaCl containing 0.1 mM EDTA for 24 h followed by further dialysis against 0.15 M NaCl for 24 h.

Fig. 6. Effect of the duration of LDL oxidation on the various products and their toxicity. LDL (1.5 mg protein/ml) was incubated with 20 μM $CuSO_4$ at 37℃ for the indicated intervals, then dialyzed against 0.15 M NaCl containing 0.1 mM EDTA for 24 h followed by further dialysis against 0.15 M NaCl for 24 h. TBARS, LPO, fluorescence intensity and cytotoxicity were determined as described under Materials and Methods. In the assay for cytotoxicity, the test medium supplemented with 2 μM $CuSO_4$ was also measured. Values in A and B represent the means of at least duplicate determinations which differed by no more than 5%. Values in C represent the means of duplicate wells which differed by no more than 5%.

Table 1. Polyunsaturated fatty acids in oxidized LDL

LDL (1.5 mg protein/ml) was incubated with 20 μM $CuSO_4$ at 37℃ for the indicated intervals. Samples were then dialyzed against 0.15 M NaCl containing 0.1 mM EDTA followed by further dialysis against 0.15 M NaCl for 24 h. Polyunsaturated fatty acids in Ox-LDL were determined as described under Materials and Methods. Values represent the means of duplicate determinations which differed by no more than 5%.

Fatty acid (μg/ml)	Incubation time 0 h	24 h	48 h
18 : 3	10.4	5.2	2.4
20 : 3	17.0	10.4	6.0
20 : 4	94.4	36.2	12.7
20 : 5	21.8	4.4	2.0
20 : 6	48.7	8.0	5.6

Fig. 1

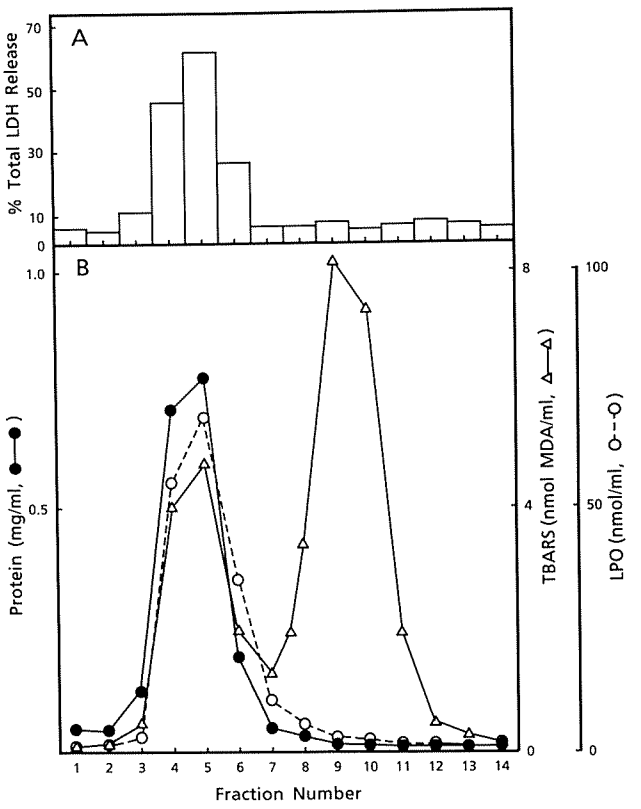

A
% Total LDH Release
70
50
30
10
0
B
1.0
0.5
Protein (mg/ml, ●—●)
8
4
0
TBARS (nmol MDA/ml, △—△)
100
50
0
LPO (nmol/ml, O--O)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fraction Number

Fig. 2

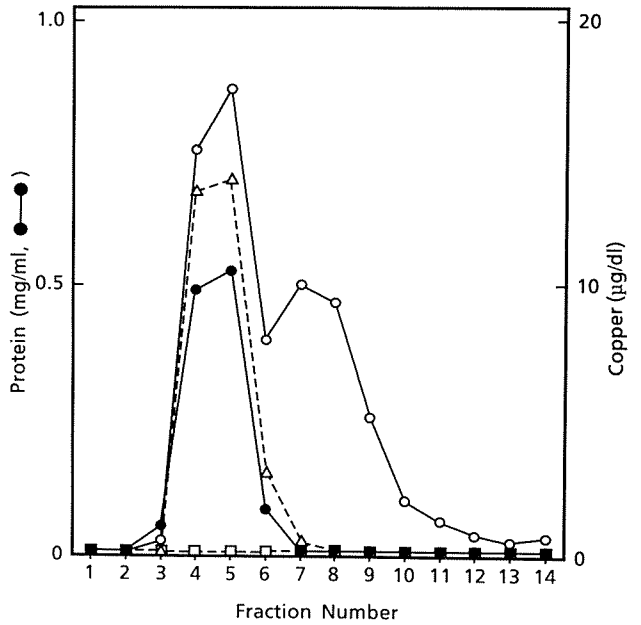

1.0
0.5
0
Protein (mg/ml, ●—●)
20
10
0
Copper (µg/dl)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fraction Number

Fig. 3

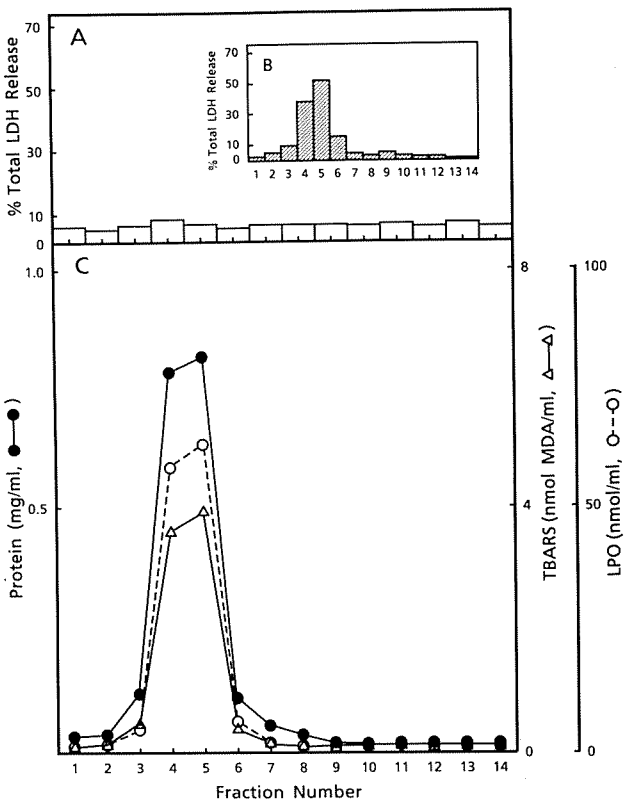

A
B
C
% Total LDH Release
% Total LDH Release
Protein (mg/ml, ●—●)
TBARS (nmol MDA/ml, △—△)
LPO (nmol/ml, O--O)
Fraction Number

Fig. 4

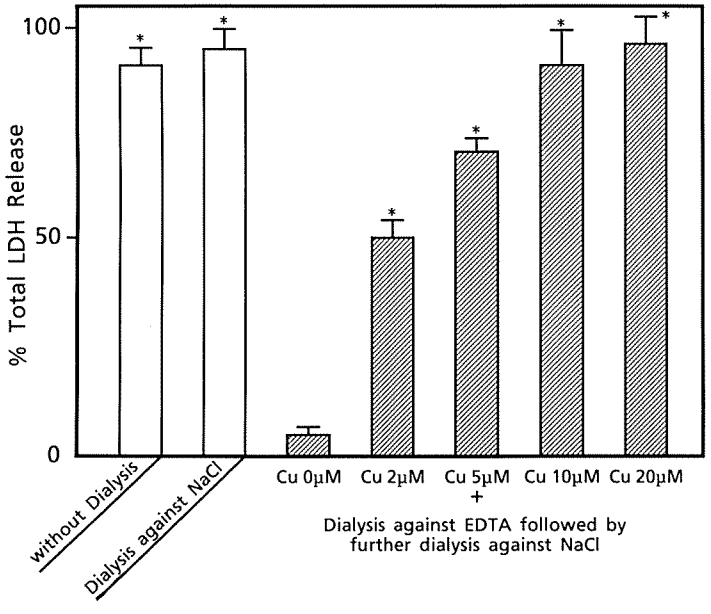

% Total LDH Release
without Dialysis
Dialysis against NaCl
Cu 0μM
Cu 2μM
Cu 5μM
+
Cu 10μM
Cu 20μM
Dialysis against EDTA followed by
further dialysis against NaCl

Fig. 5

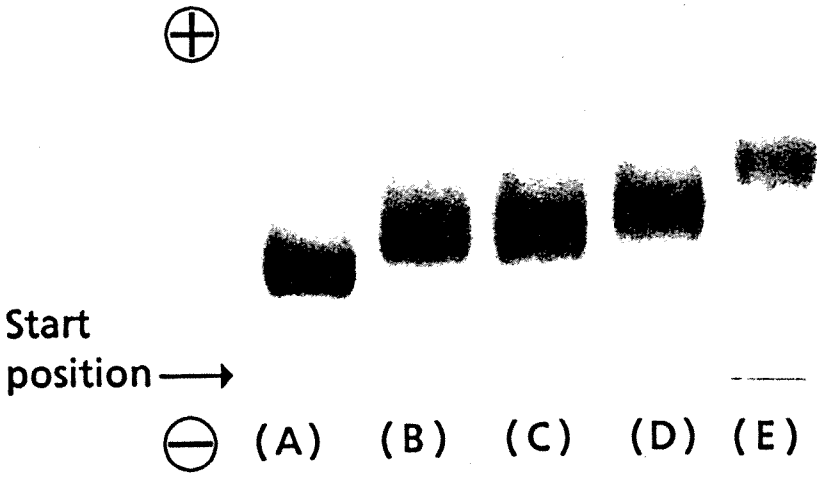
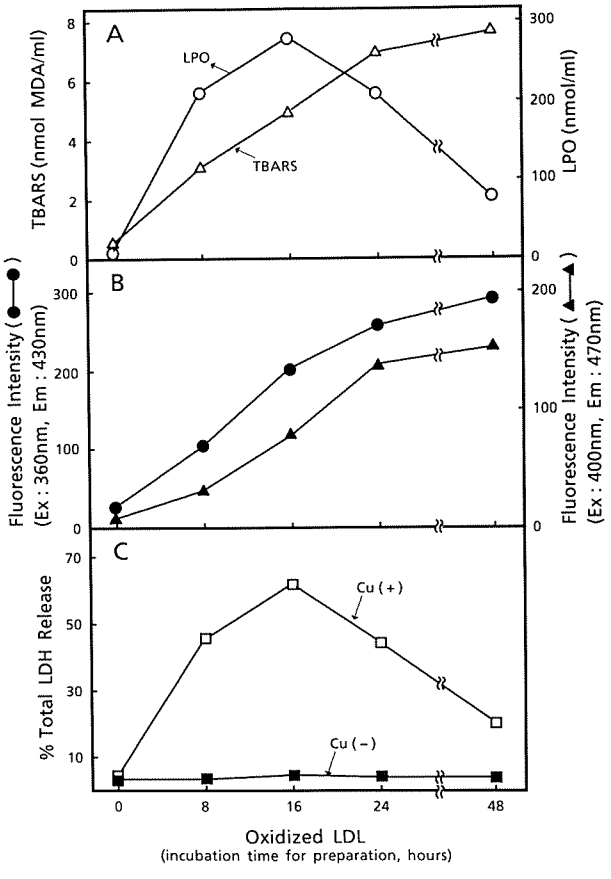

⊕
Start position ⟶
⊖ (A) (B) (C) (D) (E)

Fig. 6


A
LPO
TBARS
TBARS (nmol MDA/ml)
LPO (nmol/ml)
B
Fluorescence Intensity (●) (Ex : 360nm, Em : 430nm)
Fluorescence Intensity (▲) (Ex : 400nm, Em : 470nm)
C
Cu (+)
Cu (−)
% Total LDH Release
Oxidized LDL
(incubation time for preparation, hours)