REVISTA ESPAÑOLA DE FISIOLOGIA, 42, 99-104. 1986

Lipid Peroxidation of Low Density Lipoprotein by Human Endothelial Cells Modifies its Metabolism *in vitro*

Ll. Masana *, M. Shaikh, A. La Ville and B. Lewis

Department of Chemical Pathology and Metabolic Disorders
St. Thomas's Hospital Medical School
London

(Received on April 10, 1985)

LL. MASANA, M. SHAIKH, A. LA VILLE and B. LEWIS. *Lipid Peroxidation of Low Density Lipoprotein by Human Endothelial Cells Modifies its Metabolism* in vitro. Rev. esp. Fisiol., 42, 99-104. 1986.

Low density lipoprotein (LDL) undergoes qualitative changes when incubated with endothelial cells. Changes in LDL induced by cultured human endothelial cells were associated with release of substances reacting to thiobarbituric acid; they were prevented by addition of EDTA. Modification of LDL by human endothelium, therefore, appears to involve lipid peroxidation. Proneness of LDL to this process was indicated by its ocurrence, to a smaller extent, on incubation in the absence of endothelium. Lipid peroxidation of LDL altered its electrophoretic mobility. Modified LDL, but not native LDL, was readily catabolised by human macrophages. Conditioning by human endothelium increased the rate of fractional catabolism of LDL in rabbits. If lipid peroxidation of LDL takes place *in vivo* it may promote conversion of macrophages to lipid-laden foam cells in the developing atheromatous plaque.

Key words: Atherosclerosis, Endothelial cells, LDL, Lipid peroxidation.

Lipid peroxidation may play a causal role in certain pathological processes (5) and some authorities have implicated it more widely, in ageing, cancer, atherosclerosis and many other diseases (9, 27). In the course of studies on human atherosclerotic plaques and low density lipoprotein (LDL) extractable from them we have obtained evidence that lipid peroxidation can occur in LDL, both as a laboratory artifact and as a consequence of their exposure to human endothelial cells.

An important component of the atherosclerotic plaque is lipid containing foam cells derived from macrophages (3). LDL is believed to be a major source of the cholesteryl ester present in plaques (4) particularly in subjects with elevated levels of this lipoprotein in plasma. Yet it is unclear by what mechanisms the macrophages of the plaque acquire LDL cholesterol; unlike arterial smooth mus-

* To whom all correspondence should be addressed: Departamento de Medicina Interna, Hospital Sant Joan, Facultad Medicina Reus, Universidad de Barcelona. Reus/Tarragona (Spain).

** Presented in part to the 19th Annual Meeting of the European Society for Clinical Investigation. April 1985. Toulouse (France).

cle, fibroblasts and many other cells, in which LDL mediates its uptake, macrophages do not posses such receptors (7). However macrophages show avid receptor mediated uptake of LDL that has been modified chemically, and exposure to altered LDL can lead to their conversion to foam cells (7).

Recently a notable series of papers by HENRICKSEN *et al.* (10-12) has drawn attention to a possible basis for analogous formation of modified LDL *in vivo;* incubation of native LDL with endothelial cells yields a product with increased electrophoretic mobility that is readily taken up by macrophages. LDL extracted from atheromatous plaques has greater electrophoretic mobility than plasma LDL (14); conceivable, therefore, LDL may be modified by endothelial cells or other cells *in vivo* permitting its rapid uptake by macrophages and leading to foam cell formation.

In studies directed to the further characterisation of human plaque lipoproteins, the occurrence of lipid peroxidation of LDL was noted during incubations *in vitro.* Related studies have recently been presented (26). The present report confirms those studies which employed rabbit endothelial cells and mouse macrophages. It extends the findings by showing similar results when human cells were used; finally the effect of lipid peroxidation of LDL on its metabolism in laboratory animals *in vivo* is herein reported.

Materials and Methods

Human and rabbit LDL were isolated from plasma at density 1.019-1.063 g/ml by sequential preparative ultracentrifugation (19). They were dialysed against 5.000 volumes of saline solution at pH 7.4 without or with EDTA (1 mM). Lipoprotein-deficient serum (LPDS) was obtained from human plasma by ultracentrifugation at density 1,21 g/ml; its cholesterol concentration was about 0.08 mmol/l. Human and rabbit LDL were labelled with ^{125}I by the method of MACFARLANE(17).

Human endothelial cells were isolated from umbilical cord veins by the method described by JAFFE *et al.* (15). Cells were cultured in T-25 flasks. The culture medium was HAM F-10 supplemented with HEPES, glutamine, penicillin, streptomycin and 20 % of fetal calf serum. After 3-6 days the cells reached confluence and were ready for use.

Human monocyte-derived macrophages were obtained from whole blood, isolated as described by BOYUM (2) and plated in 60 mm Petri dishes at a concentration of 0.5×10^6 cells per dish. The medium used was RPMI-1640 supplemented with 10 % of autologous serum. After 5 days they were incubated for 18 hours in medium containing 10 % LPDS and used for the experiments.

All culture mediums were purchased from GIBCO.

Endothelial cell-conditioned LDL (EC-LDL) was prepared as described by HENRICKSEN *et al.* (11) using a concentration range of LDL of 100-200 μg protein/ml serum-free HAM F-10 medium. LDL was incubated in the presence of human endothelial cells for periods of 6-48 hour, usually 24 hours. A further aliquot of LDL was incubated in identical conditions except for the absence of cells. This is referred to as medium-incubated LDL (M-LDL). In some incubation experiments the medium was supplemented with 10 % LPDS or whole human serum. The procedure was followed using other culture media with different metal ion concentrations (Dulbecco's MEM, RPMI-1640, M-199) and saline solution. Incubations in presence of EDTA 0.1-2 mM were also carried out.

Acetylation of LDL was carried out with acetic anhydride (1).

Electrophoresis in agarose gel was performed by standard methods using sudan red to stain (23).

Thiobarbituric acid reacting substances were used as a mesure of lipid peroxidation and were assayed by the method of YAGI (29).

Degradation of LDL or modified LDL by macrophages was measured by counting ^{125}I-labelled trichloroacetic acid soluble radioactivity in the medium after incubation (6). Macrophages were incubated with varying concentrations of labelled EC-LDL, M-LDL or native LDL at 37 °C for 4 hours. In competition studies 25 μg/ml of unlabelled native LDL and acetyl-LDL were added along with 10 μg/ml of labelled LDL to the incubation mixture. Cell protein was measured by the method of LOWRY (16). Results were expressed as ng of LDL protein degraded/4 h/mg cell protein.

In kinetic studies in male New Zealand white rabbits the metabolism of native and modified LDL using a double isotope procedure was compared. Aliquots of rabbit LDL were labelled with ^{125}I or with ^{131}I respectively. The ^{125}I-LDL was incubated with endothelial cells or cell-free culture medium at a protein concentration of 60 μg/ml. ^{131}I native LDL was used as the control. Immediately before injection labelled EC-LDL, M-LDL and native LDL were dialysed overnight against 2,500 volumes of buffered saline at pH 7.4 containing 1 mM of EDTA. The amounts of thichloroacetic acid-soluble radiactivity of the samples before injection were less than 3 %. Two rabbits were injected with measured amounts of ^{125}I-EC-LDL and ^{131}I-native LDL at a time and two other rabbits with ^{125}I-M-LDL and ^{131}I-native LDL.

Blood samples were drawn at 10 and 30 minutes and at 1.5, 3, 4, 5, 7, 24 and 30 hours. ^{125}I radioactivity was counted in whole plasma. The fractional catabolic rate (FCR) was calculated by the method of MATTHEUS (18).

Statistical significance was assessed using the unpaired t test.

Results

Electrophoretic mobility. Both EC-LDL and M-LDL showed greater electrophoretic mobility than native LDL. EC-LDL mobility was 119 % greater than that of native LDL and that of M-LDL was 53 % greater than that of native LDL (fig. 1). No modification occurred when EDTA 0.5 mM was added to the medium. LDL incubated in saline showed no change. The presence of LPDS or whole plasma in the medium also inhibited modification by endothelial cells or cell-free medium. Similar modifications were induced by cell-free Dulbecco's MEM and RPMI-1640 media but not by M-199 medium.

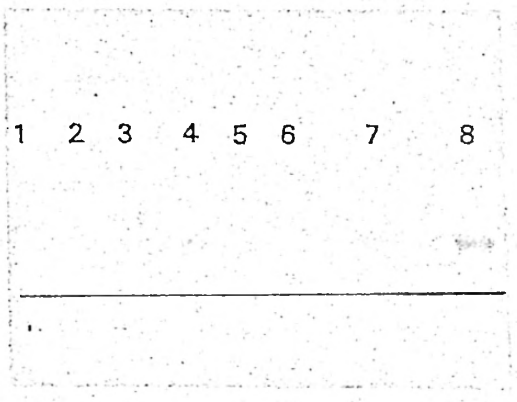

Fig. 1. *Electrophoretic mobility of native and modified low density lipoprotein.*
LDL electrophoretic mobility in agarose gel was assayed after 24 h of incubation in different conditions. After incubation with endothelial cells (2 and 5) LDL run 20.8 ± 1 mm from origin (mean ± SEM). With cell-free medium 14.5 ± 0.9 (1, 3 and 6). Control LDL (4, 7 and 8) run 9.5 ± 0.7 ($p < 0.001$ versus EC-LDL and M-LDL).

Table II. *Lipid peroxidation products (TBA-reacting substances) in the medium of modified and unmodified LDL, as malondialdehyde equivalents (means ± S.E.M.).*
Aliquots of human LDL were assayed for lipid peroxidation products after 24 h incubation in the presence of endothelial cells and with cell-free medium (CFM) and with or without EDTA.

Incubation conditions	N	nmol/ml
Control	8	1.2 ± 0.40
+ endothelial cells	6	4.1 ± 0.90*
+ cell-free medium	7	2.9 ± 0.30*
+ CFM + EDTA 0.5 mM	2	1.2 ± 0.03

* $p < 0.02$ vs control LDL.

Electrophoretic mobility was studied after incubation for periods of 6-48 hours; increased mobility appeared at 18 h and increased with duration of incubation; it was independent of the presence of antibiotics, fungizone, glutamine, HEPES, bicarbonate or glucose in the medium. No modifications were observed when samples were incubated at 4 °C in HAM F-10 medium.

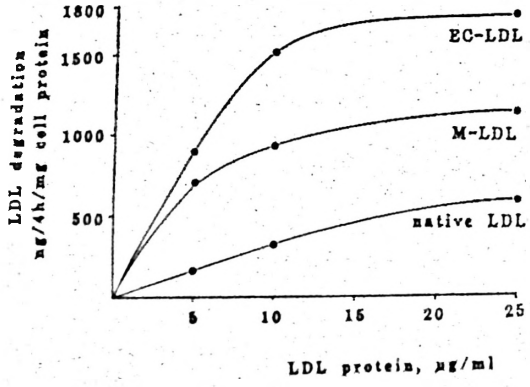

Fig. 2. *Degradation by monocyte-derived macrophages of endothelial cell-modified low density lipoprotein (EC-LDL), culture medium — modified low density lipoprotein (M-LDL) and native low density lipoprotein.*

Thiobarbituric acid reacting substances (TBA). EC-LDL and to lesser extend M-LDL had a greater amount of TBA reacting substances than native LDL (table I). Formation of TBA reacting substances was completely inhibited by the presence of EDTA during the incubation.

Density. Less than 30 % of modified LDL was present in the top fraction following ultracentrifugation at a background density of 1.063 g/ml compared with an average of 97 % for native LDL.

Degradation of LDL by macrophages (fig. 2). Degradation of EC-LDL was considerably greater than that of native LDL; M-LDL showed an intermediate rate of degradation. The degradation of EC-LDL was inhibited by the presence of acetyl-LDL (—45 %, —49 % respectively) but less by unlabelled native LDL (—11 %, —10 % respectively).

Kinetic studies in the rabbit. It was confirmed that rabbit LDL underwent modification during incubation in the presence of endothelial cells and in the presence of cell free HAM F-10 medium; rabbit EC-LDL and M-LDL increased

Table II. *Fractional catabolic rate (FCR) of ^{125}I-EC-LDL and ^{125}I-M-LDL compared with ^{131}I-native LDL (Mean±S.E.M.) of two experiments).*
Two rabbits were injected with ^{125}I-EC-LDL plus ^{131}I-native LDL and other two with ^{125}I-M-LDL plus ^{131}I-native LDL. FCR was calculated from the plasma radioactivity decay curve.

	FCR (pools/h)	% change
Rabbits 1 and 2		
EC-LDL	0.152 ± 0.019	+21
Native-LDL	0.126 ± 0.006	
Rabbits 3 and 4		
M-LDL	0.160 ± 0.009	+ 9
Native-LDL	0.147 ± 0.013	

their electrophoretic mobility by 63 % and 37 % respectively after 22 h of incubation. After injection into rabbits, the FCR of EC-LDL was 21 % greater, and that of M-LDL was 9 % greater than that of native LDL (table II). Comparing the disappearance curves, the major difference was seen to be the steeper slope of the first of two exponentials.

Discussion

The studies suggest that human endothelial cells can modify the composition of LDL by a mechanism involving lipid peroxidation. They confirm and extend observations recently reported by STEINBRECHER *et al.* (26), using rabbit endothelium. The significance of these qualitative changes in LDL is the possibility that they underlie one of the characteristic features of the atherosclerotic plaque, namely the formation of lipidladen foam cells when modified LDL is taken up by macrophages (3). LDL was shown to be susceptible to lipid peroxidation by SCHUH *et al.* (25), which was supported in the present study by occurrence of this reaction even when the lipoprotein was incubated with culture media such as HAM F-10 in the absence of cells. However the extent of lipid peroxidation was considerably enhanced in the presence of human or rabbit endothelium (26); but unlike plasma LDL the endothelium-modified product readily underwent receptor —mediated uptake by human macrophages.

Modification of LDL by endothelial conditioning was shown to involve lipid peroxidation by the generation of thiobarbituric acid reacting substances; further evidence was the inhibition of the reaction by chelation of divalent metalic ions by addition of NaEDTA to the medium. HAM F-10 induced e greater lipid peroxidation possibly because of its great Fe^{2+} and Cu^{2+} concentration. Lipid peroxidation involves the reaction of polynsaturated fatty-acids with a free radical initiator, forming a lipid —free radical intermediated that reacts in turn with oxygen (28). The process is catalyzed by Fe^{2+} and Cu^{2+} (8). It is involved in physiological conditions (27). Among disorders that predispose to atherosclerosis, diabetes has been associated with abnormal lipid peroxidation (22); and plasma obtained from men immediatly after smoking is citotoxic for cultured mesothelial and endothelial cells, producing morphological changes very similar to those caused by lipid peroxidation (24).

LDL cytitoxicity has been attributed to lipid peroxidation (13, 20).

These results suggest that modification of rabbit LDL accelerated its catabolism in rabbits, possibly reflecting its uptake by a selective pathway other than those by which LDL is cleared. Modified LDL injected into rats shows an enhanced fractional catabolic rate (21).

If lipid peroxidation is found to occur *in vivo* the present findings, like those of STEINBRECHER *et al.* (11) suggest that this process may play a role the development of the atherosclerotic plaque.

Acknowledgements

LM was supported by a postdoctoral grant from the «Sociedad Española de Medicina Interna» and Sandoz Laboratories (Sandoz-82). We are grateful to Prof. R. Taylor and Dr. S. Raju for providing umbilical cords, and to Drs. N. E. Miller and A. W. Segal for helpful discussions.

Resumen

Cuando se incuban lipoproteínas de baja densidad (LDL) en presencia de células endoteliales humanas se producen cambios cualitativos asociados a la liberación de sustancias que reaccionan con ácido tiobarbitúrico, no produciéndose si se añade EDTA al medio de cultivo, sugiriendo que se produce peroxidación lipídica de las partículas durante el

proceso. La tendencia de las LDL a su peroxidación se demuestra porque el mismo fenómeno ocurre, en menor grado, con su incubación en ausencia de células. La peroxidación lipídica de las LDL incrementa su movilidad electroforética. Las LDL modificadas son rápidamente captadas y metabolizadas por los macrófagos no ocurriendo así con las normales. Las LDL condicionadas por endotelio también muestran un aumento de su fracción catabólica en conejos.

References

1. Basu, S. K., Goldstein, J. L., Anderson, R. G. W. and Brown, M. S.: *Proc. Natl. Acad. Sci. USA*, **73**, 3178-3182, 1976.
2. Boyum, A.: *Scand. J. Clin. Lab. Invest.*, **21** (Suppl. 97), 77-89, 1968.
3. Brown, M. S. and Goldstein, J. L.: *Ann. Rev. Biochem.*, **52**, 223-261, 1983.
4. Brown, M. S., Goldstein, J. L., Krieger, M., Ho, Y. K. and Anderson, R. G. W.: *J. Cell Biol.*, **82**, 597-613, 1979.
5. Diplock, A. T.: *Phil. Trans. R. Soc. Lond.*, **B294**, 105-117, 1981.
6. Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S.: *Proc. Natl. Acad. Sci. USA*, **76**, 333-337, 1979.
7. Goldstein, J. L. and Brown, M. S.: *J. Biol. Chem.*, **249**, 5153-5162, 1974.
8. Gutteridge, J. M. C.: *FEBS Lett.*, **150**, 454-458, 1982.
9. Halliwell, B. and Gutteridge, J. M. C.: *Lancet*, **1**, 1396-1397, 1984.
10. Henricksen, B., Mahoney, E. M. and Steinberg, D.: *Proc. Natl. Acad. Sci. USA*, **78**, 6499-6503, 1981.
11. Henricksen, B., Mahoney, E. M. and Steinberg, D.: *Arteriosclerosis*, **3**, 149-159, 1983.
12. Henricksen, B., Mahoney, E. M. and Steinberg, D.: *Ann. NY. Acad. Sci.*, **401**, 102-116, 1982.
13. Hessler, J. R., Morel, D. W., Lewis, L. J. and Chisolm, G. M.: *Arteriosclerosis*, **3**, 215-222, 1983.
14. Hoff, H. F., Bradley, W. A., Helderman, C. L., Gaubatz, J. W., Karagas, M. D. and Gotto, A. M.: *Biochim. Biophys. Acta*, **573**, 361-374, 1979.
15. Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R.: *J. Clin. Invest.*, **52**, 2745-2756, 1973.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: *J. Biol. Chem.*, **193**, 265-275, 1951.
17. MacFarlane, A. S.: *Nature*, **182**, 53, 1958.
18. Matthews, C. M. E.: *Physics. Med. Biol.*, **2**, 36-53, 1957.
19. Mistry, P., Miller, N. E., Laker, M., Hazzard, W. R. and Lewis, B.: *J. Clin. Invest.*, **67**, 493-502, 1981.
20. Morel, D. W., Hessler, J. R. and Chisolm, G. M.: *J. Lipid. Res.*, **24**, 1070-1076, 1983.
21. Nagelkerke, J. F., Havekes, L., Hinsbergh, V. W. M. and Berkel, T. J. C.: *Arteriosclerosis*, **4**, 256-264, 1984.
22. Nishigaki, I., Hagihara, M., Tsunekawa, H., Maseki, M. and Yagi, K.: *Biochem. Med.*, **25**, 373-378, 1981.
23. Noble, R. P.: *J. Lipid Res.*, **9**, 693-700, 1968.
24. Noronha-Dutra, A. A. and Steen, E. M.: *Lab. Invest.*, **47**, 346-353, 1982.
25. Schuch, J., Fairclough, G. H. and Haschemeyer, R. H.: *Proc. Natl. Acad. Sci. USA*, **75**, 3137-3139, 1978.
26. Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witzum, J. L. and Steinberg, D.: *Proc. Natl. Acad. Sci. USA*, **81**, 3883-3887, 1984.
27. Tappel, A. L.: *Fed. Proc.*, **32**, 1870-1874, 1973.
28. Tappel, A. L. and Dillard, C. J.: *Fed. Proc.*, **40**, 174-178, 1981.
29. Yagi, K.: *Biochem. Med.*, **15**, 212-216, 1976.