JCI The Journal of Clinical Investigation

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J Clin Invest. 1984;74(5):1890-1894. https://doi.org/10.1172/JCI111609.

Research Article

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Rapid Publication

Iron and Copper Promote Modification of Low Density Lipoprotein by Human Arterial Smooth Muscle Cells in Culture

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Abstract. Modification of low density lipoproteins by human arterial smooth muscle cells was characterized by increased electrophoretic mobility and increased content of malondialdehyde-like oxidation products reactive with thiobarbituric acid. Lipoprotein modification was promoted by micromolar concentrations of iron or copper in the culture medium and was metal ion concentration- and time-dependent. The ability of diverse media to promote smooth muscle cell-mediated low density lipoprotein modification correlated with their iron concentration. Therefore, metal ion concentration of culture media contributes substantially to low density lipoprotein modification in vitro. Human monocyte-derived macrophages took up and esterified the cholesterol from modified low density lipoprotein more extensively than from native low density lipoprotein. Metal ion-mediated modification of low density lipoprotein may be a contributing factor to the pathogenesis of arteriosclerosis.

Introduction

Low density lipoproteins (LDL) play a central role in atherosclerosis. Elevated levels of LDL, for example due to genetic abnormalities of the LDL receptor, result in accelerated coronary artery disease (1). Macrophages and arterial smooth muscle cells have been implicated as precursors of foam cells, which are prominent components of atherosclerotic plaques (2, 3). Paradoxically, monocyte-derived macrophages take up LDL slowly in vitro and fail to accumulate large quantities of cholesterol (4). However, macrophages possess a scavenger lipoprotein receptor, distinct from the LDL receptor, that takes up modified species of lipoprotein in an unregulated manner resulting in accumulation of lipid and acquisition of characteristics of foam cells (5, 6). LDL altered chemically or by incubation with cultured endothelial or guinea pig arterial smooth muscle cells is endocytosed by this scavenger receptor (5–9).

LDL may also undergo lipid peroxidation to a form toxic to cells in culture (10). Oxidized LDL and certain chemically modified forms of LDL react with thiobarbituric acid, an index of malondialdehyde (MDA)[1] like material in the lipoprotein. The thiobarbituric acid-reacting substances (TBARS) are a measure of lipid peroxidation (11). All modified forms of LDL that are taken up by the scavenger receptor, as well as oxidized LDL, show increased mobility on electrophoresis compared with control LDL.

Contaminating trace metals may be a significant and unrecognized variable in biological oxidations (12). Transition metal ions such as Fe and Cu play an important role in catalyzing certain oxidation-reduction cycles (13). Lipid peroxidation is also known to be transition metal-dependent (14). Therefore, the present study was undertaken to evaluate the potential role of the metal ions Fe, Cu, and Zn in the modification of LDL by human arterial smooth muscle cells in culture.

Methods

Cell culture. Smooth muscle cells derived from explants of human aorta (15) were plated at 5×10^4 cells/35 × 10-mm dish and fed with Dulbecco's modified Eagle's medium (DMEM-Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum. Cells were used within 2 d of reaching confluency. Human monocytes were obtained by a combination of density gradient and counterflow cen-

Address reprint requests to Dr. Heinecke.
Received for publication 14 May 1984 and in revised form 16 August 1984.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/84/11/1890/05 $1.00
Volume 74, November 1984, 1890–1894

1. *Abbreviations used in this paper:* DMEM, Dulbecco's modified Eagle's medium; MDA, malondialdehyde; TBARS, thiobarbituric acid-reacting substances.

trifugation as previously described (16). They were plated at 4×10^5 cells/16 × 10-mm dish in DMEM supplemented with 20% autologous serum. Monocyte-macrophages were used within 14 d of plating. Resident peritoneal macrophages from Swiss-Webster mice (25–35 g) were isolated by the method of Edelson and Cohn (17). They were plated at 1×10^6 cells/16 × 10-mm dish in DMEM supplemented with 20% fetal calf serum. 2 h later, the nonadherent cells were removed by washing. The cells were placed in fresh medium containing 20% fetal calf serum and were used for experiments the next day.

Lipoproteins. Human LDL (density, 1.019–1.063 g/ml) was isolated by ultracentrifugation in a vertical rotor (Beckman VTi50; Beckman Instruments, Inc., Fullerton, CA). The LDL band was aspirated after tube slicing and washed once in a fixed angle rotor (Beckman 60 Ti; Beckman Instruments, Inc.) at density 1.063 g/ml. The LDL was then extensively dialyzed against 0.15 M NaCl and 1 mM EDTA. Acetyl LDL was prepared by the method of Basu et al. (18).

Smooth muscle cell-modified LDL was prepared as follows. Cultured arterial smooth muscle cells were preincubated for 24 h in serum-free DMEM. Cells were washed once with DMEM and incubated with 1 ml of DMEM at the indicated concentrations of LDL (225 μg LDL protein/ml for the TBARS assay and electrophoresis; 500 μg LDL protein/ml for use in the macrophage uptake experiments). At the end of incubation, the medium was collected, centrifuged at 1000 *g* for 10 min, and stored at 4°C under nitrogen until used. Cell-free controls were incubated under identical conditions except that preincubation with DMEM was omitted. In experiments where metal ion concentration in DMEM was varied, aliquots of concentrated solutions of $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, or $ZnSO_4 \cdot 7H_2O$ were added to the medium. In some experiments, various other media (Ham's F10, Ham's F12, medium 199, or RPMI 1640, all from Gibco Laboratories) were substituted for DMEM during incubation as noted.

Metal ion concentration. The Fe, Cu, and Zn concentration of media were determined by inductively coupled atomic emission spectroscopy using the method of standard additions (19).

Other methods. The TBARS assay of Buege and Aust (11) was used in modified form. 73 μg of LDL protein was present in a final volume of 1.5 cm^3. To ensure that metal ion content of the assay did not vary significantly, $CuSO_4$, $ZnSO_4$, and $FeSO_4$ were added before heating to achieve a final concentration of 87 μM. TBARS was expressed as the MDA equivalent content (nanomoles MDA per milligram LDL protein) using an extinction coefficient determined from a MDA tetramethyl acetal-generated standard curve (20). Protein content was measured by the method of Lowry et al. (21).

Macrophage uptake of control and smooth muscle cell-modified LDL was measured by incorporation of [^{14}C]oleic acid into cholesteryl ester (22). The final concentration of LDL in the medium was 100 μg protein/ml.

Agarose gel electrophoresis was carried out at pH 8.6 in barbital buffer using a Beckman Paragon system (Beckman Instruments, Inc.). Electrophoretic mobility was expressed as the ratio of the distance migrated of modified to control LDL.

Results

Modification of LDL during incubations with arterial smooth muscle cells was promoted by increasing Fe and Cu concentration of the medium (Fig. 1). No effect of Zn was found. Electrophoretic mobility and TBARS of the LDL increased at

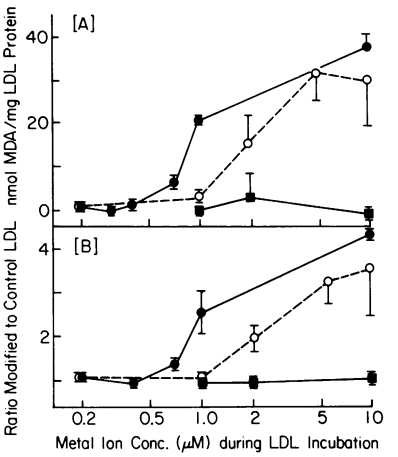

Figure 1. Effect of metal ion concentration on LDL modification by human smooth muscle cells. Cells were preincubated for 24 h in serum-free DMEM, washed once, and incubated in DMEM with LDL at the concentration of Fe, Cu, or Zn indicated. Stock DMEM is represented by 0.2 μm Fe and Cu (since the measured values were ≦0.2 μM, the lower limit of detection of the assay for these ions). (*A*) TBARS and (*B*) electrophoretic mobility were determined as described in the Methods. Each data point represents the mean±SD of two experiments with duplicate values per experiment. — ● —, Cu; - - ○ - -, Fe; — ■ —, Zn.

1 μm concentration of Cu and 2 μm concentration of Fe. LDL was unmodified following 24-h incubation with smooth muscle cells in DMEM alone, which contained undetectable levels (≦0.2 μm) of Fe and Cu. The TBARS content of control LDL was 0.8±2.3 nmol MDA/mg LDL protein with a relative mobility of 1.0±0.1, while LDL incubated in DMEM to which no metal ions were added had TBARS of 0.7±0.8 nmol MDA/mg LDL protein with a relative mobility of 1.1±0.1. In a cell-free system, Fe and Cu altered TBARS and electrophoretic mobility to a lesser degree. Further, a considerably higher concentration of Cu and Fe was required for LDL modification in the absence of cells (Fig. 2). On electrophoresis, modified LDL migrated as a single band, suggesting modification of all the LDL in the medium. There was no increase in the TBARS in the medium of cells incubated without lipoprotein at the highest concentrations (10 μm) of Fe, Cu, or Zn compared with cells incubated in DMEM alone. Desferrioxamine at 10 times the Fe concentration and butylated hydroxytoluene at 25 μM completely inhibited Fe promoted LDL modification. Linear regression analysis of the data in Figs. 1 and 2 showed a strong correlation between TBARS and electrophoretic mobility ($r = 0.977$, $P < 0.001$).

Incubation of LDL with arterial smooth muscle cells in media containing various concentrations of Fe and Cu confirmed the importance of metal ion concentration in LDL modification by smooth muscle cells (Table I). Changes in TBARS and electrophoretic mobility of LDL could be ac-

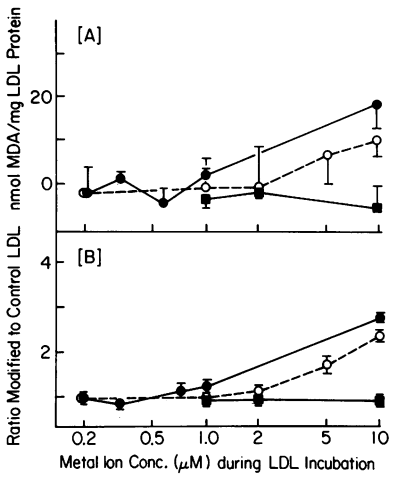

Figure 2. Effect of metal ion concentration on LDL modification in cell-free dishes. Conditions were as in Fig. 1 except that preincubation with DMEM was omitted. — ● —, Cu; - - ○ - -, Fe; — ■ —, Zn. (*A*) TBARS; (*B*) electrophoretic mobility.

counted for by the concentration of Fe in the medium. The Cu concentration in all the media was below the range where promotion of LDL modification would be expected.

The time course of LDL modification by arterial smooth muscle cells incubated in DMEM with either 1 μM Cu or 2 μM Fe was examined (Fig. 3). TBARS and electrophoretic mobility changed in parallel.

LDL modified by incubation with smooth muscle cells in the presence of 10 μM Fe or Cu stimulated cholesteryl ester formation by human monocyte-derived macrophages (Table II). LDL that was modified under identical conditions in a cell-free system also stimulated cholesteryl ester formation by macrophages, but to a lesser extent than cell-modified LDL, similar to the observed changes in TBARS and electrophoretic mobility. The addition of Fe or Cu to unmodified LDL failed to stimulate its uptake by macrophages. LDL that had been modified by smooth muscle cells in the presence of Fe or Cu also stimulated cholesterol esterification in resident mouse peritoneal macrophages (Table III).

Table I. Effect of Media Composition on LDL Modification by Human Arterial Smooth Muscle Cells

	LDL modification		Metal Ion Concentration (μM)		
Medium	Electrophoretic mobility*	TBARS‡	Fe	Cu	Zn
DMEM	1	4.5	UD§	UD	0.8±.2
RPMI 1640	1	4.1	UD	UD	0.3±.2
Ham's F10	3.1	26.9	1.7±.4	UD	0.3±.2
Ham's F12	3.3	33.5	2.2±.4	UD	3.0±.4
Medium 199	0.9	0.0	UD	UD	UD

Cells were preincubated for 24 h in serum-free DMEM, washed once, and incubated for 24 h in the indicated medium with LDL. Electrophoretic mobility, TBARS, and metal ion concentration were determined as described in the Methods.
* Ratio of migration of modified to control LDL.
‡ Nanomoles MDA per milligram LDL protein.
§ UD, undetectable; ≤0.2 μM.

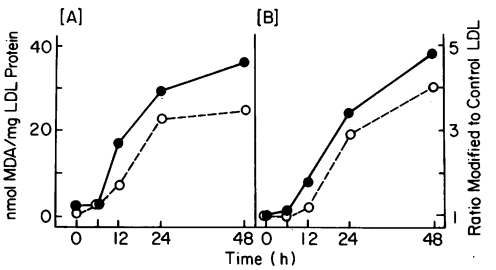

Figure 3. Time course of LDL modification by human smooth muscle cells. Cells were incubated in DMEM with LDL in the presence of either 2 μM Fe or 1 μM Cu. Conditions were as in Fig. 1. — ● —, Cu; - - ○ - -, Fe. (*A*) TBARS; (*B*) electrophoretic mobility.

Discussion

These studies demonstrate that the Fe and Cu content of the incubation medium profoundly influences the modification of

Table II. Uptake of Modified LDL by Human Monocyte-derived Macrophages

LDL	Incorporation of [^{14}C]oleate into cholesteryl ester
	nmol/mg protein
Omitted	1.2±0.1
Native	5.0±1.7
Acetylated	29.5±7.4
Smooth muscle cell-modified: medium additions	
None	4.1±0.3
Fe (10 μM)	13.3±1.2
Cu (10 μM)	17.3±0.3
Cell-free incubation:	
None	3.5±0.1
Fe (10 μM)	9.9±1.6
Cu (10 μM)	9.1±0.8

Macrophages were washed twice with DMEM and incubated with 100 μg/ml lipoprotein for 24 h. Data are mean±SD of triplicate values.

Table III. Uptake of Modified LDL by Mouse Peritoneal Macrophages

LDL	Incubation time	Incorporation of [^{14}C]oleate into cholesteryl ester
	h	*nmol/mg protein*
Omitted	—	4.2±1.3
Acetylated	—	796±193
SMC modified: additions		
None	24	11.3±0.7
	48	12.3±0.5
Fe (10 μM)	24	31.9±0.5
	48	53.1±1.1
Cu (10 μM)	24	39.3±1.5
	48	45.7±5.2

Experimental conditions were as for Table II.

LDL by human arterial smooth muscle cells. Modification, as assessed by an increase in TBARS and in electrophoretic mobility, is promoted by micromolar concentrations of either Fe or Cu. The absence of change in TBARS in the medium of cells incubated with Fe or Cu without lipoprotein suggests that non-LDL-associated lipid peroxidation did not occur. Fe-promoted LDL modification is inhibited by the chelating agent desferrioxamine, and the free radical inhibitor, butylated hydroxytoluene. Commercially available media differ widely in their content of these metal ions. This may partly explain why only certain cell types have been shown to modify LDL, since incubations have been carried out in a variety of media (9). These findings emphasize the importance of metal ion concentration of medium used in studies of LDL in vitro.

LDL modified during dialysis in the absence of antioxidants or chelating agents has been shown to be cytotoxic (10, 23). Modification was associated with increased TBARS and electrophoretic mobility, and was inhibited by EDTA. Free radical peroxidation of lipid was proposed as the mechanism of modification. Free radical-mediated mechanisms also appear to play an important role in the oxidation of LDL by endothelial cells in vitro (9, 24). Our data suggest that Fe or Cu initiate or promote a free radical process. Cu has recently been reported to mediate similar effects in the modification of LDL by endothelial cells (9).

Henriksen et al. (8) have shown that LDL modified by incubation with endothelial cells or guinea pig arterial smooth muscle cells is more rapidly degraded by macrophages than is native LDL. The LDL modified by incubation with the human smooth muscle cells which we have described is incorporated into cholesteryl ester to a greater extent than control LDL by human monocyte-derived macrophages and resident mouse peritoneal macrophages. These findings are consistent with increased uptake and degradation by macrophages of the smooth muscle cell-modified LDL. Since LDL receptor activity on mouse peritoneal macrophages is very low, the increased uptake observed with modified LDL in these cells is consistent with uptake by another class of receptors such as the scavenger receptor, which has been shown to mediate the uptake of endothelial cell-modified LDL (7, 9). Because biologically modified forms of LDL may result in cholesteryl ester accumulation in macrophages in vivo, the importance of Fe and Cu in accelerating atherogenesis needs to be determined.

Acknowledgments

We thank Linda Baker, Karen Tittle, and Thuy Nguyen for their excellent technical assistance, and Ann Ferguson for skillfully typing the manuscript.

This work was supported in part by National Institute of Health grants HL 18645, HL 30086, AM 02456, AM 07247, and AI 07763. Dr. Chait is an Established Investigator of the American Heart Association. Dr. Rosen is the recipient of Research Career Development Award AI 00368 from National Institute of Allergy and Infectious Diseases.

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