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Oxidative Modification of Low-Density Lipoprotein by the Human Hepatoma Cell Line HepG2

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The human hepatoblastoma cell line HepG2 is a liver model commonly used for lipid metabolism studies. Numerous cell types have been found to oxidize low-density lipoprotein (LDL) but, to our knowledge, the effects of HepG2 cells on LDL have not been investigated. We found that LDL is modified by HepG2 cells through a peroxidative mechanism, as judged by an increase in TBARS content (which was prevented in the presence of the antioxidants vitamin E, 2,6-di-*tert*-butyl-cresol and probucol), increased degradation by J774 macrophages, decreased internalization by MRC5 fibroblasts, and aggregation of apo B. Aspirin and allopurinol, which inhibit cyclooxygenase and xanthine-oxidase activities, respectively, had no effect on HepG2-induced LDL modification, and neither did catalase, which dismutates hydrogen peroxide; or mannitol, which scavenges hydroxyl radicals. In contrast, superoxide dismutase, a superoxide anion scavenger, and glutamate and threonine, which alter cellular cystine uptake, prevented LDL modifications, as did the removal of cysteine/cystine from the culture medium. Oxidation of LDL by HepG2 cells might thus involve superoxide anion production and/or thiol metabolism.

Keywords: Low density lipoprotein, modified LDL, lipid peroxidation, human hepatoma cell line, liver

Abbreviations: Apo, apolipoprotein; BHT, 2,6-di-*tert*-butyl-cresol; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulphoxide; EDTA, Ethylenediamine tetraacetic acid; ETYA, 5,8,11,14 eicosatetraynoic acid; FCS, Fetal calf serum; FPLC, Fast Protein Liquid Chromatography; HDL, high-density lipoprotein; HPLC, high-pressure liquid chromatography; LDL, low-density lipoprotein; PBS, phosphate buffer saline; pBPB, parabromophenacyl bromide; REM, relative electrophoretic mobility; SDS-PAGE, Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; SOD, superoxide dismutase; TBARS, Thiobarbituric acid-reactive substances

INTRODUCTION

HepG2 is a liver cell line derived from a human hepatoblastoma.[1,2] These cells express many liver-specific epithelial cell functions, including functions related to lipid metabolism.[3,4] HepG2 cells synthesize free and esterified cholesterol and various apolipoproteins (e.g. apolipoprotein B100), giving rise to plasma-like lipoproteins. They can take up and internalize cholesterol and

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cholesteryl-ester associated with chylomicron remnants, VLDL (very-low-density lipoprotein), LDL (low-density lipoprotein) and HDL (high-density lipoprotein). Receptors related to LRP receptors could contribute to chylomicron remnant metabolism by HepG2 cells.[5] Apo B/E receptors of the HepG2 plasma membrane bind LDL prior to their internalization and degradation through a non saturable low-efficiency transport system or a saturable high-efficiency system. LDL uptake via the high efficiency system inhibits receptor and HMG CoA reductase expression, inhibiting cholesterol synthesis. No information is available on oxidative modifications of LDL by HepG2 cells.

LDL oxidation has been shown to take a prominent part in the pathogenesis of atherosclerosis. It increases LDL uptake by macrophages,[6,7] contributing to their conversion into foam cells, and appears to involve the four major cell types within atherosclerotic lesions. Cultured rabbit aortic endothelial cells,[6,7] human umbilical vein endothelial cells,[7] human vascular smooth muscle cells,[8] human lymphocytes[9] and macrophages[10] all modify LDL, as do circulating human monocytes and polymorphonuclear cells,[11] rat stimulated mast cells,[12] human platelets[13] and mesangial cells.[14] Data on LDL oxidation by bovine aortic endothelial cells and human skin fibroblasts are contradictory. Henriksen[7] *et al.* found that the former had little effect, but Morgan[15] *et al.* recently reported that they modified LDL after longer incubation times. Fibroblasts were initially considered not to oxidize LDL,[7,16] but Steinbrecher[17] *et al.* have shown that these cells produce superoxide anion and might thus do so. We investigated whether HepG2 cells can oxidize LDL.

MATERIALS AND METHODS

LDL Isolation

Venous blood was drawn into Vacutainer tubes containing 1.7 mM EDTA and centrifuged at 2300 × *g* for 15 min at 4°C. After ultracentrifugation for 24 h at 45,000 rpm and 4°C in a L90 ultracentrifuge (Beckman, Palo Alto, USA) using a 70.1 Ti rotor, the infranatant was collected, pooled and adjusted to a density of 1.063 g/ml, recentrifuged as before and LDL was collected. It was centrifuged again for 24 h in KBr (Prolabo, Paris, France), density 1.063 g/ml, pH 7.4, containing 1 mM EDTA (Sigma, St-Louis, USA), dialysed three times for 8 h against 0.01 M Tris-HCl (Sigma) buffer pH 7.4 containing 1 mM EDTA, it was filtered on a 0.2 µm membrane (Sartorius, Göttingen, Germany). It was kept in the dark at 4°C for two weeks.

Cell Culture

HepG2 cells (The American Type Culture Collection, Rockville, Maryland, USA) were routinely cultured in DMEM (BioWhittaker, Verriers, Belgium) containing 4.5 g/l glucose and supplemented with 10% (v/v) FCS (Boehringer, Mannheim, Germany), 2 mM glutamin, 100 U/ml penicillin and 100 µg/ml streptomycin (Techgen, Les Ulis, France) in a humidified atmosphere of 5% CO_2, 95% air. Cells were subcultured every 5–7 days at a density of $3 \times 10^6/75$ cm^2 flask (Falcon, Beckton Dickinson). Medium was changed every 2–3 days.

To study LDL oxidation, HepG2 cells were detached by a 0.05% Trypsin-0.02% EDTA solution (Techgen) and 2×10^5 cells in 1 ml of Ham F10 medium (Gibco, Paisley, UK) containing 10% FCS, 15 mM HEPES buffer (Techgen), 2 mM glutamin, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Techgen) were seeded on 2 cm^2 plastic wells (Falcon), at 37°C in 5% CO_2. Ham F10 medium contains 25 mg/l cysteine, which was shown to autoxidize to cystine during storage.[18]

MRC5 human lung fetal line fibroblasts (Biomerieux, Marcy l'Etoile, France) were cultured in 35-mm Nunc (Roskilde, Denmark) Petri dishes in DMEM (BioWhittaker) containing 1 g/l

glucose, 2 mM glutamin, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS.

J774 murine line macrophages (The American Type Culture Collection, were cultured in 35-mm Nunc (Roskilde, Denmark) Petri dishes containing RPMI medium (BioWhittaker) supplemented with 25 mM HEPES buffer (pH 7.4), 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated FCS.

Experiments were performed with confluent cells.

Modification of LDL by HepG2 Cells

LDL was extensively dialysed against 0.02 M phosphate buffer (Prolabo) pH 7.4 containing 0.15 M NaCl (Prolabo). Cells were washed three times with Ham F10 medium to remove serum proteins. Growth-control wells were filled with complete Ham F10 medium. LDL was incubated (0.2 mg LDL protein/ml) for 48 h with HepG2 cells or in cell-free wells (control LDL) at 37°C in humidified air/CO_2 (95:5). The culture medium (1 ml/well) consisted of Ham F10 medium supplemented with 15 mM HEPES buffer, 2 mM glutamin, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The reaction was stopped with 10 µl BHT (Fluka, Buchs, Switzerland) (2 mM in methanol) and 10 µl EDTA (100 mM in phosphate buffer saline). Aliquots of the supernatant were taken for the measurement of thiobarbituric acid reactive substances (TBARS) and the rest was dialysed three times for 8 h against 0.01 M Tris-HCl buffer pH 7.4 containing 1 mM EDTA.

Modification of LDL by $CuSO_4$

LDL was extensively dialysed against 0.02 M phosphate buffer pH 7.4 containing 0.15 M NaCl. LDL diluted to 1 mg LDL protein/ml in this buffer was incubated 24 h at 37°C in the presence of 50 µM $CuSO_4$ (Prolabo).

Effect of Inhibitors

We studied the effects of BHT, probucol (generous gift from Merrel-Dow), vitamin E, SOD from bovine liver, allopurinol, catalase, aspirin, ETYA, pBPB, mannitol (Sigma), threonine and glutamate (Merck, Darmstadt, Germany) on 24 h oxidation of LDL by HepG2 cells. Ham F10 medium without cysteine (Gibco) was used as well. When DMSO (Merck) was used as solvent (for BHT, probucol, vitamin E, allopurinol, aspirin, ETYA and pBPB), the stock solutions were prepared so that the final concentration in the wells was 1% (v/v), which was found to have no effect on LDL modification (data not shown).

Thiobarbituric Acid-Reactive Substances (TBARS) Assay

The TBARS content of LDL was determined using a kit manufactured by Sobioda (Grenoble, France) according to a modified version of Yagi's assay.[19] Culture supernatant (100 µl) was incubated with 750 µl of solution containing thiobarbituric acid and perchloric acid for 60 min at 95°C. After stopping the reaction in an ice-bath, MDA was extracted in 2 ml butanol-1. TBA-MDA complex was determined fluorimetrically at an excitation wavelength of 532 nm and an emission wavelength of 553 nm.

The cell-specific effect of inhibitors on TBARS production was appreciated to the following index:

$$100 - 100\frac{Ic^{+} - Io^{+}}{Ic^{-} - Io^{-}}$$

Ic+: TBARS (nmol/mg LDL protein) in wells containing cells and inhibitor.
Io+: TBARS (nmol/mg LDL protein) in cell-free wells containing inhibitor.
Ic^{-}: TBARS (nmol/mg LDL protein) in wells containing cells and no inhibitor.
Io^{-}: TBARS (nmol/mg LDL protein) in cell-free wells containing no inhibitor.

An increasing index showed an increasing capacity for a substance to prevent HepG2 cell-specific production of TBARS, the theoretical maximal effect corresponding to an index of 100%. When the index was found to be equal to zero, molecules were considered to have no inhibitory effect on cell-specific production of TBARS.

Fast Protein-Liquid Chromatographic (FPLC) Separation

Electronegativity of LDL modified by HepG2 cells was studied using ion-exchange chromatography method as described by our laboratory.[20] Chromatographic system is manufactured by Pharmacia (Uppsala, Sweden) and consists of an LCC-500 programmer controlling two P-500 pumps. Two buffers were used: buffer A, 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA; buffer B, 1 M NaCl in buffer A. The sample was filtered through a 0.2-μm filter, introduced via a 0.5-ml loop onto a mono Q HR 5/5 column and eluted at 1 ml/min by a linear gradient of 0–10% buffer B during the first 10 min, followed by a multistep gradient: 10 to 15 min, 20% buffer B; 16 to 20 min, 30%; 21 to 25 min, 40%; 26 to 30 min, 50%; 31 to 35 min, 60%; 36 to 40 min, 100%; and 41 to 45 min, 0%. The effluent was monitored by means of a single path ultraviolet monitor at 280 nm. Under the described chromatographic conditions, two fractions A and B were resolved from the supernatant containing LDL incubated without cells and three fractions A, B and C after incubation with HepG2 cells, where fraction A was the least electronegative and fraction C the most electronegative one. Each fraction was quantified according to the area of the corresponding peak, calculated as a percentage of the total area represented by all three fractions.

Vitamin E Assay

Vitamin E was determined by means of HPLC.[21] A 20-μl aliquot was injected onto a Merck Lichrosorb RP-18 column with a methanol mobile phase. The detector was set at 292 nm. DL-vitamin E (Sigma) was used as standard.

Agarose Gel Electrophoresis

Universal electrophoresis film agarose was used in the Ciba Corning (Le Vezinet, France) electrophoresis system. The sample size was 2.4 μg protein, and the time chosen for electrophoretic migration was 40 min. REM (relative electrophoretic mobility) was defined as the ratio of migration distance of cell-modified LDL to native LDL.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

0.08 mg of LDL protein solutions were extracted by addition of 2 ml of ether. After vortexing, the mixture was centrifugated at 1800 × *g* for 10 min to separate the two phases and the supernatant was removed. The samples were diluted (1:1) in the following buffer: 10 mM Tris, pH 8.0 containing 1mM EDTA, 2.5% sodium dodecyl sulfate, 5% 2-mercaptoethanol and 0.01% bromophenol blue. Then, they were separated by SDS-PAGE using the Pharmacia Phast system with Phastgel gradient 4–15. We used the separation technique and silver staining method given by the manufacturer.

LDL Internalization and Degradation

LDL labeling was performed with Na ^{125}I (Amersham, Le Ulis, France) as described by Bilheimer *et al.*.[22] Specific radioactivity was 157 cpm/ng of LDL protein, and free iodine in the preparation was 0.7%. MRC5 cells were pretreated for 24 h in DMEM devoid of lipoprotein and supplemented with the serum substitute Ultroser G. MRC5 and J774 cells were washed. Binding and internalization of control and cell modified LDL were studied on MRC5 cells, and degradation on J774 cells, according to Goldstein and Brown.[23]

The results are expressed in ng LDL protein per mg cell protein.

Superoxide Anion Assay

Superoxide anion production was determined according to a technique described by Steinbrecher *et al.*,[17] based on the superoxide dismutase-inhibitable reduction of ferricytochrome *c*.[24]

Cells were grown for 48 h in 2-cm^2 wells (Falcon) in Ham F10 medium (Gibco) specially formulated without Phenol red containing or not cysteine, supplemented with FCS, glutamin and antibiotics. They were washed three times with medium devoid of FCS, and then 1.2 ml of the same medium containing 20 μM ferricytochrome *c* (Sigma) were added. After 10, 20, 40 and 60 min incubation at 37°C, the reaction was stopped in an ice-bath, and the absorbance of the medium was read at 550 nm. Controls without cells and containing 100 U/ml SOD from bovine liver (Sigma) were used. Superoxide production was calculated using a molar extinction coefficient of 20 $mmol^{-1}$ cm^{-1} for reduced cytochrome *c*. The superoxide concentration is expressed as nmol per mg cell protein.

Protein Assay

Total protein was measured by using Peterson's method[25] with bovine serum albumin as standard.

Statistical Analysis

Statistical comparison of data was carried out using an impaired Student's *t*-test. These analyses were performed by the use of the StatView II program for the MacIntosh IIsi.

RESULTS

Influence of Various Parameters on LDL Modification by HepG2 Cells

We established optimal conditions for peroxidative modification of LDL by HepG2 cells using TBARS determination. We tested two culture media (Ham F10 and DMEM), four incubation times (6, 12, 24 and 48 h), four cell densities (25,000, 50,000, 75,000 and 100,000 cells/cm^2) and four LDL concentrations (0.05, 0.10, 0.20 and 0.40 mg LDL protein/ml).

Oxidation of LDL by HepG2 cells was apparent after 12 h of incubation (Fig. 1). The TBARS content of LDL increased from 1.0 nmol/mg LDL protein before incubation to 27.0 nmol/mg LDL protein after 48 h incubation in the presence of cells, compared to 6.2 nmol/mg LDL protein in the absence of cells. The vitamin E content of LDL was fully consumed after 48 h incubation in the cell-free wells and after only 12 h in the presence of HepG2 cells. As first observed with an endothelial cell model,[26] DMEM did not allow significant modifications of LDL by HepG2 cells (without cells: 1.70 ± 0.15 nmol/mg LDL protein; with cells: 1.75 ± 0.20 nmol/mg LDL protein, n = 3) after 48 h incubation.

In Ham F10 medium, TBARS content of LDL increased with a growing cellular density and a decreasing LDL concentration (Fig. 2). For example, after 48 h incubation in the presence of 100,000 cells/cm^2, the TBARS production due to cell-mediated oxidation was 4.94, 12.1, 15.5 and 16.8 nmol/mg LDL protein for concentrations of 0.40, 0.20, 0.10 and 0.05 mg LDL protein/ml, respectively. In subsequent experiments, a density of 100,000 cells/ml/cm^2 and a concentration of 0.20 mg LDL protein/ml were routinely used.

Characterization of LDL Modified by HepG2 Cells

Electronegativity of LDL modified by HepG2 cells was studied by both agarose gel electrophoresis and ion-exchange chromatography. Agarose gel electrophoresis (Fig. 3) showed increased electronegativity. After 48 h of incubation in Ham F10 medium, the relative electrophoretic mobility of LDL in cell-free medium was 2.00. It reached 2.62 after incubation in the presence of 100,000 cells/cm^2. In ion-exchange

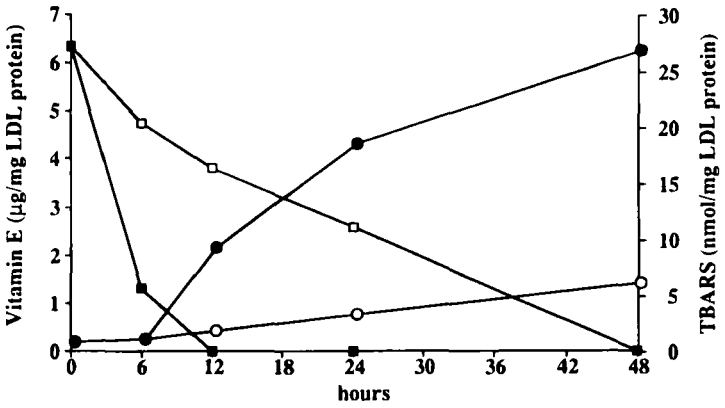

FIGURE 1 Influence of incubation time on cellular modification of LDL. Cells (2×10^5) were incubated in 2-cm^2 wells for 6, 12, 24 and 48 h, then rinsed with FCS-free Ham F10 medium. LDL in PBS was diluted to 0.20 mg LDL protein/ml in this medium and added (1 ml per well) to the cells (cell +) or to control cell-free wells (cell –). Oxidation was stopped by adding 1 mM EDTA and 0.02 mM BHT (final concentrations), and the TBARS and vitamin E contents in the supernatant were measured. (□) vitamin E in cell (–) wells, (■) vitamin E in cell (+) wells, (○) TBARS in cell (–) wells, (●) TBARS in cell (+) wells. Data are values from one experiment.

chromatography (Fig. 4), the minor fraction B in native LDL (Fig. 4a) became the major fraction after 24 h incubation in cell-free wells (Fig. 4b), and reached 100% in the presence of 100,000 cells/cm^2 (Fig. 4c). The influence of LDL concentration and cell density on FPLC chromatographic patterns was also studied (Fig. 5). After

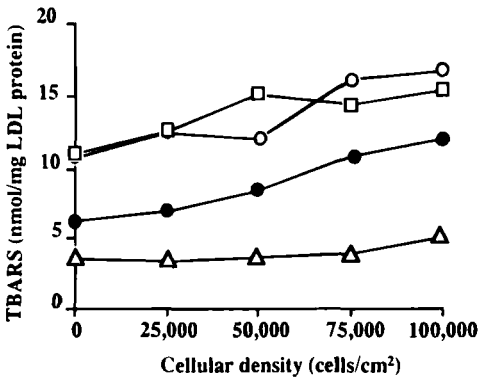

FIGURE 2 Influence of cell density and LDL protein concentration on TBARS production. Increasing cell densities were incubated in 2-cm^2 wells for 24 h, then rinsed with FCS-free Ham F10 medium. LDL dialysed against PBS was diluted to 0.05 (○), 0.10 (□), 0.20 (●) and 0.40 (△) mg LDL protein/ml in this medium. TBARS content was measured after stopping oxidation by adding 1 mM EDTA and 0.02 mM BHT (final concentrations). Values are the means of duplicate wells (CV% < 8) of one representative experiment.

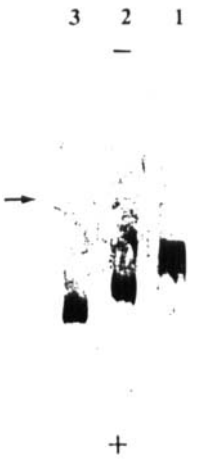

FIGURE 3 Cellular modification of LDL assessed by agarose gel electrophoresis. LDL (0.2 mg LDL protein/ml) was incubated for 48 h in 2-cm^2 wells in Ham F10 medium in the absence (lane 2) or presence of 200,000 cells (lane 3). Oxidation was stopped by the addition of 1 mM EDTA and 0.02 mM BHT (final concentrations). LDL was concentrated by ultracentrifugation. The sample size was 2.4 µg LDL protein. Native LDL were set in lane 1. The arrow indicates the origin. REM (relative electrophoretic mobility) was defined as the ratio of the migration distance of cell-modified LDL to that of native LDL. After 48 h incubation in Ham F10 medium, the REM of LDL in cell-free wells was 2.00. It reached 2.62 after incubation in the presence of 100,000 cells/cm^2.

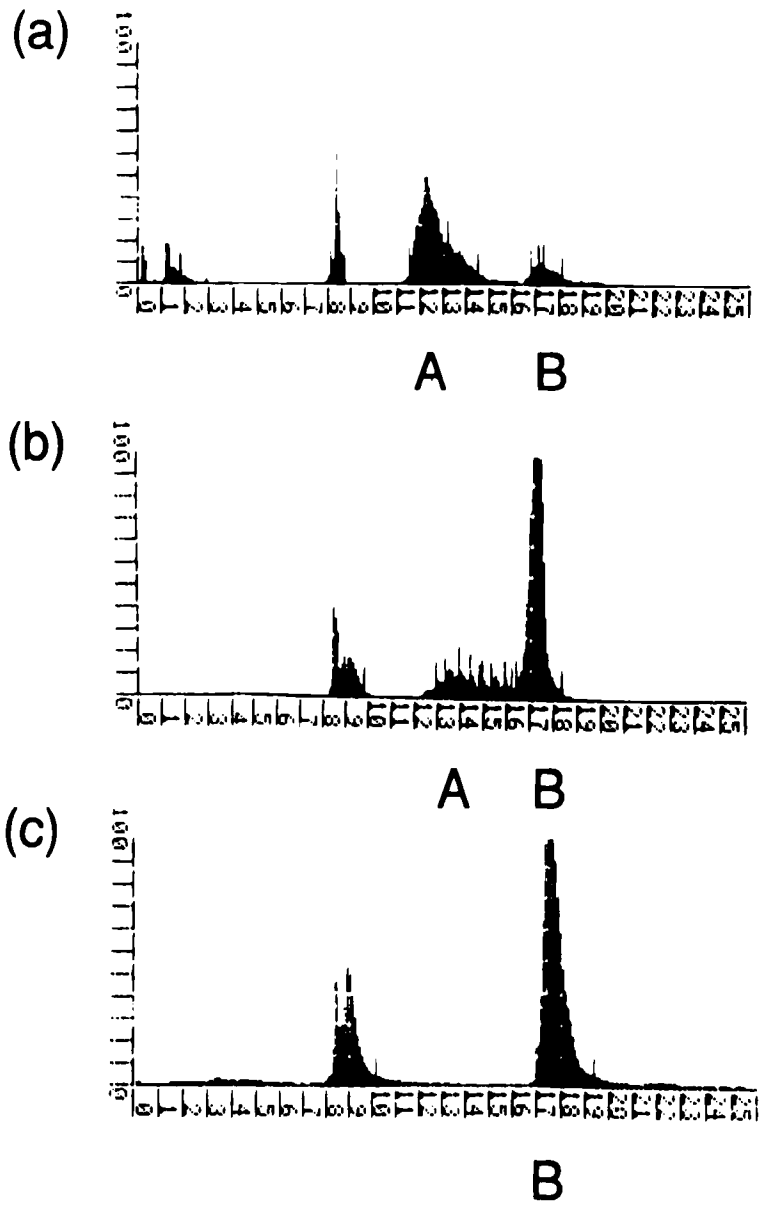

FIGURE 4 Cellular modification of LDL assessed by ion-exchange chromatography on an FPLC system. Cells (2×10^5) were incubated in 2-cm^2 wells for 24 h, then rinsed with FCS-free Ham F10 medium. LDL in PBS was diluted to 0.20 mg LDL protein/ml in this medium and added (1 ml per well) to the cell-containing or cell-free wells. Oxidation was stopped by adding 1 mM EDTA and 0.02 mM BHT (final concentrations). After dialysis against Tris-HCl buffer pH 7.4 containing 1 mM EDTA, the sample was filtered and injected into the FPLC system. Cell oxidative activity was exhibited by a decrease in the proportion of the least electronegative fraction (A) and an increase in that of the most electronegative fraction (B). Chromatograms are (a) native LDL, (b) LDL incubated in the absence of cells, (c) LDL incubated in the presence of cells.

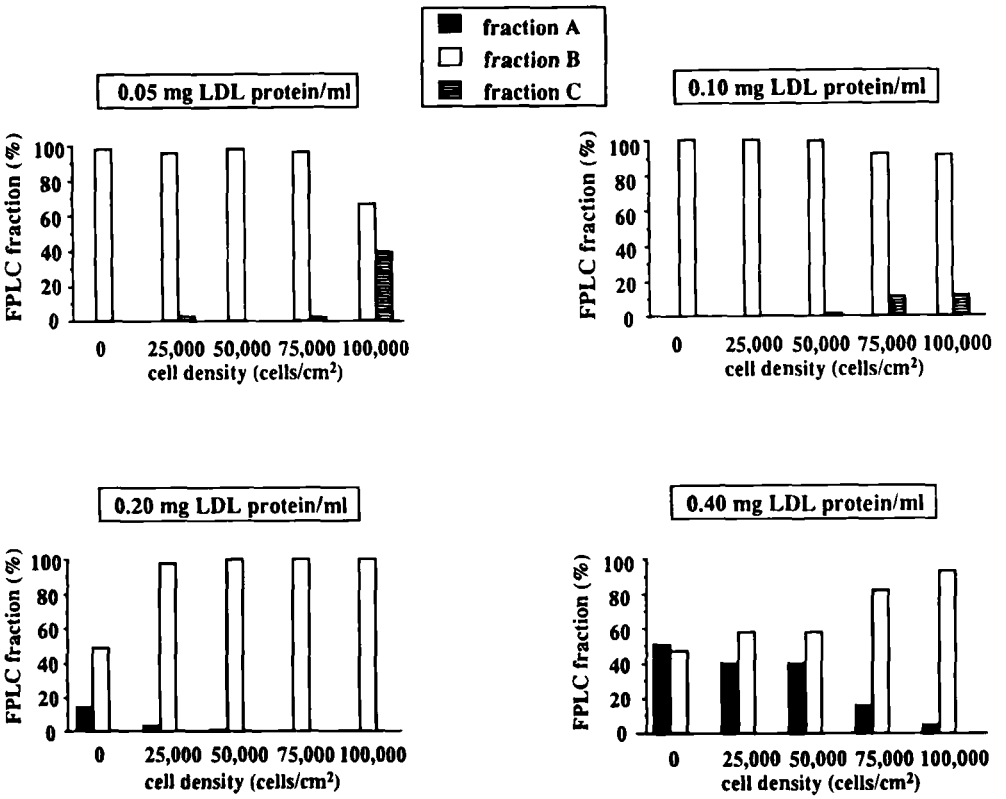

FIGURE 5 Influence of cell density and LDL protein concentration on separation by ion-exchange chromatography on an FPLC system. Cells (0.5, 1.0, 1.5 and 2.0 × 10^5) were incubated in 2-cm^2 wells for 24 h, then rinsed with FCS-free Ham F10 medium. LDL in PBS was diluted to 0.05, 0.10, 0.20 and 0.40 mg LDL protein/ml in this medium and added (1 ml per well) to cell-containing or cell-free wells. Oxidation was stopped by adding 1 mM EDTA and 0.02 mM BHT (final concentrations). After dialysis against Tris-HCl buffer pH 7.4 containing 1 mM EDTA, the sample was filtered and injected into the FPLC system. Each fraction was quantified according to the area of the corresponding peak, calculated as a percentage of the overall area of the peaks on the chromatogram. Cell oxidative activity was exhibited by a disappearance of the least electronegative fraction (A) and the appearance of the most electronegative fraction (C). Data are the values of one representative experiment.

24 h incubation, with an LDL concentration of 0.40 mg LDL protein/ml, fraction B increased from 48% to 95% with increasing cell density. With an LDL concentration of 0.20 mg LDL protein/ml, fraction B reached 100% and fraction A disappeared rapidly with increasing cell density. With an LDL concentration of 0.10 mg LDL protein/ml, fraction C appeared at a cell density of 50,000 cells/cm^2 and reached 9% with 100,000 cells/cm^2. Finally, with an LDL concentration of 0.05 mg LDL protein/ml, a peak of 32% fraction C was observed.

SDS-PAGE (Fig. 6) showed a main band of apo B-100 in native LDL (lane 3). After 48 h of incubation of LDL in cell-free wells (lane 1), the appearance of both lower and higher molecular weight fragments was observed. After incubation in the presence of cells (lane 4), some aggregates appeared and some low-molecular-weight fragments disappeared. Compared to native LDL (lane 3), control copper oxidized LDL (lane 2) showed apo B-100 with lower density, and many aggregates. Internalization and degradation of LDL incubated 48 h in the presence and in absence of cells were studied (Fig. 7). After 48 h incubation in the presence of cells, a significant increase ($p < 0.001$) of 45% in J774 line macrophage degradation compared with the control

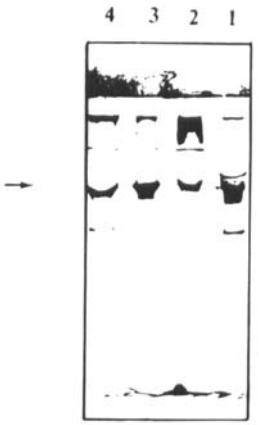

FIGURE 6 Cellular modification of LDL assessed by SDS-PAGE. LDL was prepared as described for agarose gel electrophoresis (see legend to Fig. 3). Native LDL was set in lane 3, Ham F10-modified LDL in lane 1, HepG2 cell-modified LDL in lane 4 and Cu-oxidized control LDL in lane 2. The arrow indicates the position of native apo B. A main apo B-100 band was observed in native LDL (lane 3). After 48 h incubation of LDL in cell-free wells (lane 1), native apo B-100 disappeared in parallel to the appearance of both lower and higher molecular weight fragments. After incubation in the presence of cells (lane 4), some low-molecular-weight fragments disappeared and some aggregates were seen in the stacking gel. Compared to native LDL (lane 3), control copper-oxidized LDL (lane 2) contained less apo B-100 and many aggregates.

LDL was observed. In the same experimental conditions, internalization by MRC5 line fibroblasts decreased of 12% ($p < 0.001$).

Effect of Various Inhibitors on LDL Modification by HepG2 Cells

After incubation of LDL in the presence of cells, TBARS content and electronegativity increased, and apo B was aggregated; these data are in favor of LDL peroxidation. We used antioxidants to confirm this hypothesis. After 24 h incubation in the presence of 100,000 cells/cm^2, TBARS production in LDL was inhibited by vitamin E, BHT and probucol, and chromatographic patterns exhibited less electronegative fractions (Table I).

The most informative data obtained in the study of the influence of inhibitor concentration are shown in Table I. BHT and vitamin E, which are frequently used as free radical scavengers[10,11,26–29] were tested. The chain-breaking antioxidant BHT, added at half the usual concentration[10,11,26,28] (0.02 mM) exerted a very strong inhibitory effect on LDL oxidation by HepG2 cells. A similar inhibition of TBARS production was reached with 0.05 mM vitamin E, a very high concentration,[30] although it was less effective according to the FPLC separation. Catalase, which dismutates hydrogen peroxide, and mannitol, which scavenges hydroxyl radicals, had no significant effect. On the other hand, 100 U/ml SOD, which is

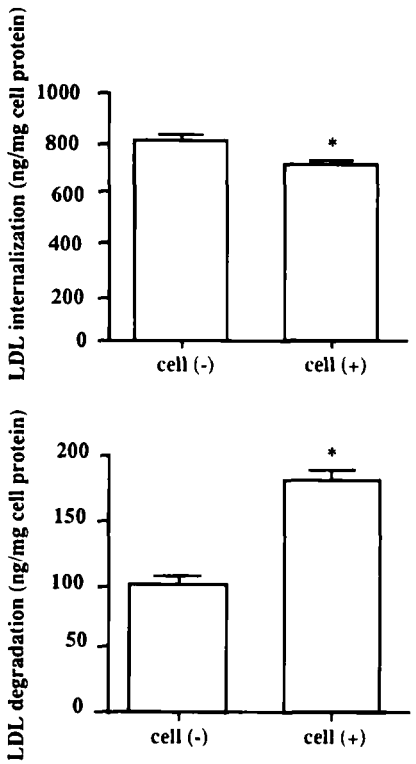

FIGURE 7 Internalization and degradation of LDL modified by HepG2 cells. ^{125}I labelled LDL (0.20 mg LDL protein/ml) was incubated for 48 h in 2-cm^2 wells in Ham F10 medium in the presence or absence of 200,000 cells. The supernatant was collected and incubated for 4 h at 37°C with MRC5 fibroblasts and J774 macrophages. Internalization was measured on the MRC5 fibroblasts pellet after rinsing with NaCl 0.9%. The rate of degradation of ^{125}I labelled LDL was estimated in the supernatant by measuring radioactive non iodide trichloracetic acid-soluble degradation products. Values are the mean ± SEM of 5 wells with the same LDL preparation. * $p < 0.001$ vs. cell (–) (Student's unpaired t test).

TABLE I Effect of Various Inhibitors on LDL Modification by HepG2 Cells

	TBARS[a] inhibition index (%)	FPLC fractions[b] Cell (–) A	B	C	Cell (+) A	B	C
Experiment 1							
Control without DMSO		5	95	—	—	90	10
Control with DMSO		8	92	—	—	93	7
Aspirin (1 mM)	0	—	100	—	—	93	7
Allopurinol (0.2 mM)	0	2	98	—	—	91	9
SOD (100 U/ml)	36	15	85	—	19	81	—
Threonine (20 mM)	58	85	15	—	33	67	—
Mannitol (5 mM)	17	6	94	—	—	89	11
Catalase (400 UI/ml)	0	9	91	—	—	93	7
Experiment 2							
Control without DMSO		—	100	—	—	90	10
Control with DMSO		3	97	—	—	91	9
Cysteine-free medium	81	40	60	—	62	38	—
Glutamate (2.5 mM)	78	15	85	—	—	100	—
Vitamin E (0.05 mM)	82	57	43	—	—	99	1
BHT (0.01 mM)	88	40	60	—	75	25	—
Probucol (0.01 mM)	79	42	58	—	—	100	—
pBPB (0.01 mM)				Cytotoxic effect			
ETYA (0.02 mM)				Cytotoxic effect			

LDL (0.20 mg LDL protein/ml) was incubated for 24 h in FCS-free Ham F10 medium (1 ml per well) containing various inhibitors in the presence of cells (100,000/cm^2). Oxidation was stopped by the addition of 1 mM EDTA and 0.02 mM BHT (final concentrations). The supernatant was collected, and TBARS content was assayed and FPLC separation was performed.

(a) TBARS content of duplicate-well supernatants was measured and the following inhibition index was calculated:

$$100 - 100\frac{Ic^+ - Io^+}{Ic^- - Io^-}, \quad \text{where}$$

Ic^+: TBARS (nmol/mg LDL protein) in wells containing cells and inhibitor.
Io^+: TBARS (nmol/mg LDL protein) in cell-free wells containing inhibitor.
Ic^-: TBARS (nmol/mg LDL proteins) in wells containing cells and no inhibitor.
Io^-: TBARS (nmol/mg LDL protein) in cell-free wells containing no inhibitor.

The higher the index, the greater the capacity of the compound to prevent HepG2 cell-specific production of TBARS.

(b) FPLC separation resolved three fractions (A, B, C). 100% corresponds to the overall area of the peaks, i.e. the total value of the three fractions. Each fraction was quantified according to the area of the corresponding peak, calculated as a percentage of this value. LDL oxidation was shown by a decrease in the proportion of the least electronegative fraction (A), an increase in that of a more electronegative fraction (B) and the appearance of the most electronegative fraction (C) after incubation in the presence of cells. Results are the percentages of each fraction. Inhibitors preventing LDL oxidation led to a return to less-electronegative fractions. Ion-exchange chromatography was performed on cell-free wells to appreciate the non cell-specific effect of inhibitors on LDL oxidation.

known not to have total inhibitory effect on superoxide anion production[17] was effective: TBARS production was inhibited of 36%, and 19% of fraction A were recovered in FPLC chromatogram. In order to appreciate the role of cysteine/cystine in superoxide-mediated modification of LDL by HepG2 cells, both TBARS production assay and ion-exchange chromatography separation after incubation in cysteine-free medium were performed. TBARS content of LDL was found to decrease of 81%, and 62% of chromatographic fraction A were resolved. Inhibitors of cystine

uptake were also used. 2.5 mM glutamate had a strong effect on LDL modification by HepG2 cells. In the presence of 20 mM threonine, a substantial inhibition was moreover observed. To confirm the role of superoxide anion on LDL modification by HepG2 cells, the production of this radical by these cells was assessed (Fig. 8). In the presence of cysteine, cumulative superoxide production was 4.3 ± 0.6, 5.7 ± 0.7, 9.3 ± 0.5 and 13.5 ± 0.7 nmol/mg cell protein (m ± SEM) after 10, 20, 40 and 60 min, respectively. In the absence of cysteine, superoxide anion production was lower. The maximal value observed was 6.8 ± 0.5 nmol/mg cell protein after 40 min incubation. In addition, superoxide dismutase at a concentration of 100 U/ml inhibited superoxide production in the presence of cysteine after 20, 40 and 60 min incubation. Inhibition was also observed in the absence of cysteine, but with longer incubation times. The involvement of enzymatic mechanisms was then considered. As assessed by phase-contrast microscopy, ETYA and pBPB were obviously cytotoxic to HepG2 cells at the concentrations used. The role of 15 lipoxygenase and phospholipase A_2 could not thus be explored. Aspirin and allopurinol, which inhibit cyclooxygenase and the xanthine oxidase/xanthine dehydrogenase system, respectively, did not prevent the production of TBARS and did not modify chromatographic patterns.

DISCUSSION

Our aim was to investigate whether HepG2 cells could oxidize LDL, and if so, to study the underlying mechanisms. TBARS content was markedly increased after 12, 24 and 48 h of incubation in the presence of HepG2 cells. In addition, LDL incubation with HepG2 cells increased their electrophoretic mobility. Ion-exchange chromatography using an FPLC system showed an increase in negative charge. The increase in TBARS content and in negative charge were dependent on the number of cells and the LDL protein concentration: the higher the cell density and the lower the LDL concentration, the stronger the LDL modifications. The alteration of electrophoretic mobility and chromatographic behavior could be due to an increase in the net negative charge of apo B resulting from both neutralization of positive ε amino groups on lysine by lipoperoxidation products, and formation of dicarboxylic amino acids following proline and histidine degradation.[30] Polyacrylamide gel electrophoresis demonstrated a complete loss of intact apolipoprotein B-100 and the appearance of fragments with high molecular weights, pointing to the cleavage and aggregation of apolipoprotein. These processes could explain the decreased internalization by MRC5 fibroblasts correspond-

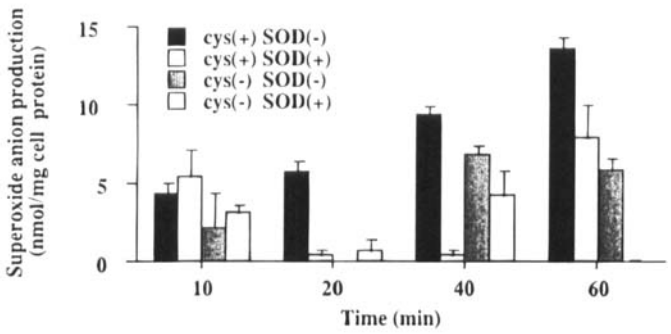

FIGURE 8 Superoxide production by HepG2 cells. 3.5×10^5 cells were incubated in 2-cm^2 wells in complete Ham F10 medium containing cysteine for 48 h at 37°C; they were then washed with serum-free, Phenol-red-free Ham F10 medium with (cys +) or without cysteine (cys–), with (SOD +) or without (SOD –) 100 U/ml superoxide dismutase. 1.2 ml of the same medium containing 20 μM ferricytochrome *c* was added. Controls without cells were used. After incubation at 37°C, the reaction was stopped in an ice bath and absorbance was determined at 550 nm. Values are the mean ± SEM of triplicate wells in one representative experiment.

ing to altered recognition by the apo B/E receptor, and the increased degradation by J774 line macrophages resulting from better recognition by the scavenger receptor.[31] The TBARS content of LDL incubated with HepG2 cells fell strongly in the presence of the chain-breaking antioxidants BHT and vitamin E, and the lipid-lowering drug probucol.

These data suggested that HepG2 cells modified LDL through a peroxidative process, as do endothelial cells (from human umbilical vein[29] and rabbit artery[26]), arterial smooth muscle cells (of human,[8,27] monkey[27] and bovine[29] origin), mouse macrophages[10] and human monocytes,[11,28] total mononuclear cells[32] and neutrophils.[11,33] Reductive inactivators of lipoxygenases such as NDGA and quercetin have been shown to inhibit cellular LDL modifications, an effect attributed to their general antioxidant capacity.[34–37] In contrast, specific inhibitors of 5-lipoxygenase have no inhibitory action.[36,37] ETYA is often used as a lipoxygenase inhibitor because, despite its lack of specificity, it has the avantage of not being an antioxidant. ETYA inhibits LDL modification by rabbit aortic endothelial cells,[34] human monocytes[35] and mouse macrophages.[38] However, it is cytotoxic for HepG2 cells, as for other cell models,[36–37] like 0.04 mM pBPB on endothelial cells.[39] The latter was cytotoxic at 0.02 mM on HepG2 cells, and lower concentrations do not inhibit the hydrolysis of phosphatidylcholine.[39] Experiments with aspirin at the classical concentration of 1 mM[34] ruled out a role of cyclooxygenase in LDL modification by HepG2 cells, as previously described with other cell types. The presence of divalent metal ions such as Fe II and Cu II enhanced LDL oxidation by rabbit aortic endothelial cells,[26] human arterial smooth muscle cells[8] and activated human monocytes.[40] Transition metal ions appeared to play a crucial role in the LDL modifications by HepG2 cells too, as the latter modified LDL in Ham F10 medium (containing 0.01 μM cupric ion and 3.00 μM iron), but not in DMEM, which lacks copper. We recently confirmed that the capacity of Ham F10 medium to oxidize LDL in the presence or absence of cells correlated strongly with the presence of metal ions and not with its electrochemical properties.[41] As transition metals facilitate free-radical reactions,[42] these findings support the possibility that LDL modification by HepG2 cells occurs via a free-radical-mediated process involving lipid peroxidation. Free-radical processes may be facilitated by transition metal complexes, which convert superoxide anion into more reactive chemical species and promote lipid hydroperoxide breakdown to form further free radicals. Redox metals take part in superoxide-dependent modifications of LDL by cells.[31] As previously observed by Steinbrecher *et al.*,[17] SOD partially inhibited cytochrome *c* reduction by superoxide anion. This phenomenon could be attributed to the conversion of superoxide anion to a dismutase-insensitive reductant in the medium, or to the secretion of other reductants by cells, such as thiols. Superoxide radical has been proposed to play a role in LDL oxidation by endothelial cells,[17] smooth muscle cells,[27] monocytes[28] and macrophages,[43] on the basis of correlations between the measure of extracellular release of superoxide anion and rates of LDL oxidation, and the inhibition by superoxide dismutase of cellular modification of LDL. In the same way, LDL modification by HepG2 cells in Ham F10 medium probably involves this reactive oxygen species, as we observed significant superoxide anion production and a strong inhibitory effect of SOD. However, Jessup *et al.*[44] doubted the involvement of this anion, as its generation in cell-free medium does not modify LDL[45,46] and because SOD inhibits copper-mediated LDL oxidation. They used the NADPH-oxidase inhibitor diphenylene-iodinium, that is not an antioxidant but has variable inhibitory effects and is cytotoxic for some cells. Thus, SOD is certainly not the ideal superoxide anion inhibitor. However, LDL oxidation by HepG2 cells required transition metal ions and

was inhibited by superoxide dismutase but not by the hydrogen peroxide inhibitor catalase, or by the hydroxyl radical inhibitor mannitol; apo B was aggregated and LDL metabolism by fibroblasts and macrophages were both altered. Heinecke *et al.*[46] have made similar observations on LDL modification by L-cystine. Thus, as suggested by these authors[47] using arterial smooth muscle cells, HepG2 cells could modify LDL by converting L-cystine to a thiol, which would promote LDL oxidation by a superoxide-dependent pathway. This hypothesis is supported by the fact that HepG2 production of superoxide anion fell more than 50% in cysteine-free medium. These thiol compounds were also involved in LDL modification by cells, as we observed a decreased TBARS content and an alteration of LDL electronegativity (FPLC) after incubation of LDL in cysteine-free medium. LDL oxidation was also significantly inhibited when cystine uptake was abrogated by 2.5 mM glutamate (a specific competitor for cystine uptake by the Xc^- system,[48] that can prevent LDL oxidation by endothelial cells[17]) and 20 mM threonine, which was recently shown to block this uptake by HepG2 cells.[49] As described in smooth muscle cells,[47] HepG2 cells could take up L-cystine and reduce it intracellularly to a thiol such as L-cysteine. The latter would then be exported into the extracellular medium and could be responsible for the extracellular reduction of O_2 to superoxide anion. This phenomenon could also explain the dismutase-insensitive reduction of cytochrome *c* by HepG2 cells. Extracellular thiol autoxidation and simultaneous reduction of O_2 to superoxide anion could give rise to thiyl radicals and reduced metal ions, both of which can support lipid peroxidation independently of superoxide anion production. The role of the xanthine oxidase/xanthine dehydrogenase system, as a source of hepatic intracellular superoxide,[50] was excluded by the lack of effect of allopurinol. Finally, superoxide anion is known to be one contributor to LDL peroxidation, formed during thiol autoxidation in the presence of metal ions, along with thiyl and hydroxyl radicals, and with H_2O_2.[51] Although a lipid phase hydroxy-radical mediated process cannot be ruled out, on the basis of these results, we conclude that HepG2 cells modify LDL through a peroxidative process involving superoxide anion production and/or thiol metabolism.

LDL oxidation *in vivo* is believed to occur in the subendothelial space of the artery wall[52] and to give rise to immunogenic LDL. Autoantibodies against epitopes of oxidized LDL have been detected in serum. Some authors[53–55] but not others[56–57] found an increased titer of these antibodies in patients with cardiovascular disease. In addition, aging does not correlate with the antibody titer.[54,58–60] Tissues other than the artery wall might also take part in this process, partially explaining the contradictory results described above. In our opinion, the liver is a potential site of oxidized LDL formation: (i) the liver is a major site of lipid metabolism. For example, it removes from plasma both remnant particles resulting from the catabolism of triglyceride-rich lipoproteins and LDL through the apo B/E receptor. Moreover, it is an important site of synthesis for lipoproteins such as HDL and VLDL.[61] (ii) Hepatic lipid peroxidation by free radicals has been associated with liver disease, essentially tumors and toxic injury by alcohol, anticancer drugs and halogenated hydrocarbides.[62] (iii) Panasenko *et al.*,[63,64] using the perfused rabbit liver model, have shown that the liver can synthesize and secrete lipoproteins with an increased content of lipid peroxidation products. They suggested that circulating oxidized LDL could originate in the liver. (iv) A strong increase in titers of plasma antibodies against oxidized LDL has recently been described in patients with liver diseases.[65]

The liver is a key organ in the detoxification of oxidized LDL, a role that seems to be at odds with its possible role in their production. Indeed, the liver is believed to be a major protection system against arterial insult by atherogenic particles.[66–69] It consists of four cell types: hepatocytes, and endothelial, Küpffer and fat-storing

cells. Rat liver endothelial cells express scavenger receptors that interact with human acetylated LDL and copper-oxidized LDL.[66] However, rat Küpffer cells appear to be more efficient in removing copper-oxidized LDL, which is more representative of *in vivo* modified LDL. They take up cholesteryl esters from oxidized LDL, and hydrolyze them to free cholesterol before conversion to bile acids and secretion into bile.[68] They also catabolize LDL oxidatively modified by treatment with a superoxide-generating system.[69] Parenchymal cells also appear to take part in detoxifying the body of modified LDL, as acetylated and copper-oxidized LDL were recognized by specific sites. However, LDL oxidized *in vitro* by copper may not be the best model for pathophysiologically modified LDL. Indeed, Nagelkerke *et al.*[70] reported a major role of rat liver endothelial cells in removing LDL modified biologically by human endothelial cells, and a minor role of parenchymal cells. Thus, hepatocytes do not appear to be strongly involved in removing LDL modified by cells, particularly hepatocytes themselves. If they did so, they would share with macrophages[10] the ability to both modify and scavenge LDL.

Owing to the limited availability of human liver tissue, the human hepatoma cell line HepG2 is commonly used for studies of human lipid metabolism. Our results raise the possibility that liver contributes to the modification of LDL *in vivo*. Whether the liver plays a direct role in LDL oxidation remains to be established.

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