LDL Oxidation and Human Placental Trophoblast and Macrophage Cytotoxicity

(44224)

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Abstract. **We have previously shown that LDL is oxidized by placental cells in primary tissue culture and that this process causes cytotoxicity proportional to LDL oxidation in the presence of sex steroid hormones. Here we describe further experiments linking LDL oxidation to placental cell cytotoxicity. Trophoblast and macrophages were isolated from healthy elective caesarean section placentas by enzymatic digestion and separated by centrifugation on a 40% Percoll gradient and maintained in primary culture for up to 5 days. LDL was oxidized by exposure to 5 μ*M* $CuCl_2$, cells were incubated in the absence of albumen to favor oxidation, and cytotoxicity was measured by ^{51}Cr release from prelabelled cells and cell protein content. Native LDL was oxidized by both cell types with a 10%–50% increase in lipid peroxides and an approximately 4-fold increase in TBARS formation. Increasing concentrations of native LDL and oxidized-LDL increased ^{51}Cr release and diminished protein content in cells incubated in HAM's F-10 medium. Addition of 5 μ*M* Cu^{2+} augmented cytotoxicity of LDL in macrophage and trophoblast culture, but more in macrophages than trophoblast. Cytotoxicity was diminished by adding 0.001–0.1 mM EDTA to the system, diminishing ^{51}Cr release from 91 ± 0.5 to 40.8 ± 1.0% in macrophages and 54.2 ± 1.2 to 33.1 ± 1.3% in trophoblast (*P* < 0.001 in both instances). Similarly, the absence of transition metal ion in culture (MEM medium) blocked an increase in ^{51}Cr release compared to its presence (HAM's F-10 medium). An antioxidant, butylated hydroxytoluene, diminished ^{51}Cr release and LDL electrophoretic mobility in HAM's F-10 medium in placental macrophage culture. LDL oxidation injures placental macrophages and trophoblast, the former more than the latter. The process is LDL- and transition metal ion–dependent and is inhibited by antioxidant. This model of LDL oxidation and placental cell damage *in vitro* provides a basis for studying mechanisms of placental dysfunction and senescence in human pregnancy.** [P.S.E.B.M. 1998, Vol 217]

Lipoproteins have both physiological and pathophysiological effects on placental function. Physiological effects on the placenta include delivery of VLDL triglyceride fatty acids by the lipoprotein lipase reaction (1) and delivery of LDL and HDL_2 cholesterol *via* LDL receptor dependent and independent mechanisms respectively (2). HDL_3 reverses this process by removing cholesterol from placental cells (2). More recently the multifunctional LDL receptor–related protein (LRP) has been found in placental trophoblast (3). This receptor takes up chylomicron remnants, α_2 macroglobulin and other molecules (3). Lipoprotein delivery to placental cells is believed to support fetal growth (1) and endocrine steroidogenesis (2).

Regarding pathophysiological effects, placental trophoblast and macrophages contain scavenger receptors that take up modified lipoproteins such as acetylated and oxidized LDL (4). Modification of LDL by acetylation, oxidation, or glycosylation diminishes progesterone secretion from cultured placental trophoblast (4). Enhanced lipid oxidation has been reported in normal, diabetic and toxemic pregnancy (5–12) (see Ref. 13 for review), and antibodies to

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oxidized LDL are increased in toxemic pregnancy (14). Most recently we have found that placental trophoblast and macrophages accelerate LDL oxidation in the presence of Cu^{++} and that the induced cytotoxicity is proportional to the extent of LDL oxidation when perturbed by the addition of sex steroid hormones (15).

The purpose of the present investigation is to document further the association of LDL oxidation with cytotoxicity of placental cells by demonstrating a relationship to LDL concentration, metal ion dependency, and antioxidant inhibition. If this association can be established, a case for placental cell damage by oxidized LDL can be made as has been done for oxidized LDL and atherogenesis (16–21). These results can also provide an experimental model for studying the possible role of oxidized LDL in the pathogenesis of trophoblast disorders in pregnancy. Some of these results have been previously reported in abstract form (22).

Methods

Isolation of Placental Cells. Placentas were obtained from pregnant women undergoing elective cesarean section at 38–42 weeks gestation. As described previously (1), the placentas were placed on ice immediately after delivery for cell isolation, which commenced within 30 min of delivery. After removal of associated membranes, small (2–3 cm^3) pieces of placenta were obtained, avoiding areas of calcification, and rinsed several times with cold saline. Placental tissue was carefully teased from vascular stroma and coarsely minced, rinsed with HAM's F-10 medium (Flow Laboratories Inc., McLean, VA), and saline, and transferred to a 250-ml flask containing an enzyme solution of 0.05%–0.1% collagenase class II (Worthington Biochemical, Freehold, NJ), 0.05% hyaluronidase type III (Sigma Chemical Co., St. Louis, MO), 4 u/ml of DNA-ase type I (Sigma Chemical Co), and 5% fetal calf serum (FCS) (Gibco, Grand Island, NY) in HAM's F-10 culture medium with glutamine. The mixture was incubated in a shaking bath at 37°C for 60 min. Placental debris was then allowed to settle, and the supernatant was filtered through three layers of sterile gauze into 50-ml tubes and centrifuged at 200*g* for 7 min. The pellets were washed twice with HAM's F-10 medium and pooled. This cellular suspension, containing 50–150 × 10^6 cells, then was layered in two tubes containing 30 ml of a 40% Percoll gradient (Pharmacia, Piscataway, NJ) and centrifuged at 800*g* for 15 min. Three bands of cells were isolated: one at d:1.033–1.048 g/ml (middle band), another at d:1.077–1.100 g/ml (bottom band), and a third at d > 1.140 g/ml containing red cells (1). Cells from the middle and bottom bands were washed twice with HAM's F-10 medium and counted with a hemocytometer yielding 20–50 × 10^6 cells in each bands. Cell viability was more than 95% as assayed by trypan blue exclusion. Using monoclonal antibodies and immunostaining, we have shown that the middle band of cells is composed of 80%–85% trophoblast with the remainder macrophages, and the bottom band of cells is about 95% macrophages with the remainder trophoblasts (1, 4).

Placental Cell Culture. Middle- and bottom-band cells were suspended in serum-free AIM-5 medium (Gibco, Grand Island, NY) to achieve a final concentration of 0.5–1 × 10^6 cells/ml. One ml of this cell solution was incubated in each well of a 24-well culture plate at 37°C (Falcon-Becton-Dickinson Labware, Lincoln Park, NJ) with 95% air, 5% CO_2. After 48 hr, cultured cells were attached to the plates, and syncytia were observed in cultures from the middle band. Culture media were changed daily.

Lipoprotein Isolation and Oxidation. LDL (d:1.019–1.063 g/ml) was isolated from EDTA-treated plasma by sequential ultracentrifugation as previously described (1, 2). The lipoproteins were dialyzed against 150 m*M* NaCl, 1 m*M* EDTA, pH 7.4, stored at 4°C under N_2 in the dark and used within 3 weeks. Lipoprotein concentration is reported as protein content determined by the Lowry method (23). EDTA-free LDL and HDL were prepared by dialysis against phosphate buffered saline (PBS), pH 7.4 (v:v, 1:1000) at 4°C for 24 hr. Oxidized LDL (Ox-LDL) was prepared by adding 5 μ*M* $CuCl_2$ to EDTA-free LDL (1 mg of LDL protein/ml) and incubating at 37°C under sterile conditions for 20 hr.

Radiochromium Release Assay and Cell Protein Content. Placental macrophages and trophoblast were incubated in AIM-5 serum-free medium for 3 days. AIM-5 medium was then removed and 1 ml of HAM's F-10 or MEM medium containing ^{51}Cr 1 μCi/ml was added to each well. After 3 hr incubation, the cells were gently washed four times with the same medium. One ml of fresh culture medium was then added to each well, and placental cell cultures were incubated under different experimental conditions at 37°C for 20 hr unless otherwise stated. The medium was removed after the incubation, and the cells were washed twice with 1 ml of 150 m*M* NaCl. The washing fluid was collected and pooled with the medium to count the radioactivity (cpm_s). Cells and cell components floating in the medium were collected by centrifugation, and the radioactivity in this pellet counted (cpm_p). Cells remaining attached in the wells were dissolved in 1 ml of 0.1 *M* NaOH, and the radioactivity was counted (cpm_c). The following expression representing the percentage of chromium released was then calculated:

$$\text{Total } ^{51}\text{Cr released (\%)}: \frac{cpm_s + cpm_p}{cpm_s + cpm_p + cpm_c} \times 100$$

The baseline ^{51}Cr release tends to be higher in these studies than in immortal cell lines because of the lesser viability of cells in primary culture and the poorer adherence of the placental cells to plastic than other types of cells. As another measure of cell damage, cell protein was measured in the cells that remained attached to culture wells after careful washing using the Lowry method (23).

Assays of Lipoprotein Oxidation. Thiobarbituric

acid reacting substances (TBARS) were determined as previously described (24). Malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxypropane was used as a standard. Lipid peroxides were determined using a commercial reagent from Merck as described by El-Saadani *et al.* (25). Agarose gel electrophoresis was performed to determine changes in the electrophoretic mobility (26).

Statistics. Statistical comparisons were performed using the paired and unpaired student *t* tests (27).

Results

To show that placental trophoblast and macrophages accelerate LDL oxidation in cell culture, native LDL was incubated in HAM's F-10 medium without serum or antioxidants in the presence and absence of cells. HAM's F-10 medium contains the transition metal ions Fe^{++} (2.98 μM) and Cu^{++} (9.6 nM). Measures of LDL oxidation are presented in Table I. Lipid peroxide formation increased from baseline to a maximum of 55%–93% in trophoblast and 86%–91% in macrophages. Lipid peroxide formation increased with an increasing number of cells in three instances but increased and then declined in the fourth. TBARS formation increased more strikingly to a maximum of 3.7- to 4.0-fold, increasing with the number of both trophoblast and macrophages. These results show that LDL is oxidized by placental trophoblast and macrophages and are consistent with previous studies where TBARS increased with length of incubation whereas lipid peroxides rose then declined with time, reflecting the intermediate status of lipid peroxide in LDL oxidation (15).

To determine if LDL oxidation is injurious to cells, increasing concentrations of native LDL were added to trophoblast and macrophages pre-labelled with ^{51}Cr at 37°C for 20 hr and incubated in HAM's F-10 medium in the absence of albumen, to favor LDL oxidation. Addition of LDL at increasing concentrations heightened ^{51}Cr release and decreased cell protein content in a concentration-dependent manner in both macrophage and trophoblast cultures (Fig. 1). In this experiment, macrophages were more sensitive than trophoblast to the cytotoxic effect of LDL with ^{51}Cr release beginning at LDL concentrations of 25–50 μg/ml in macrophages and around 100 μg/ml in trophoblast. A similar result was observed in a second experiment where cells were incubated at native LDL protein concentrations of 0, 50, 100, 200 and 300 μg/ml, respectively. In trophoblast, ^{51}Cr release was 37, 37, 38.5, 50 and 70% and in macrophages ^{51}Cr release was again more exuberant: 42, 50, 70, 97 and 95%, respectively.

The relative cytotoxic effect of LDL in cultures of macrophages and trophoblast was replicated in six experiments at a medium LDL concentration of 200 μg/ml (Table II). Macrophage and trophoblast cytotoxicity increased approximately 2-fold with the addition of native LDL, with macrophages showing greater susceptibility to cytotoxicity than trophoblast ($P < 0.02$).

To determine if previously oxidized LDL (Ox-LDL) could cause placental cell cytotoxicity, LDL was oxidized in the presence of 5 μM Cu^{++} for 20 hr and then incubated with placental cells in HAM's F-10 medium under conditions identical to the above experiments with native LDL. As shown in Figure 1, ^{51}Cr release increased and cell-protein content decreased in cultures of macrophages and trophoblast incubated with increasing concentrations of Ox-LDL. The cytotoxic effect of LDL was again concentration dependent, and the concentrations of LDL initiating cytotoxicity in macrophages (25–50 μg/ml) were again lower than in trophoblast (50–100 μg/ml). The cytotoxic effects of Ox-LDL were replicated in seven experiments with Ox-LDL added at final medium concentrations of 200 μg/ml LDL protein (Table II). In these experiments, Ox-LDL significantly increased the percentage of ^{51}Cr released from baseline in macrophages (about 65%) and trophoblast (about 35%) (both $P < 0.001$ from non-LDL containing control wells). Macrophages were again more sensitive to the stress of adding oxidized LDL than was the trophoblast ($P < 0.001$). In both placental cell types, the cytotoxicity of

Table I. Effects of Placental Cells on LDL Oxidation in HAM's F-10 Medium for 3–6 hr*

	× 10^6 cells/culture well				
	0	0.3	0.6	1.0	1.5
Lipid Peroxides (nmole/100 μl)					
Trophoblast					
Exp 1	2.96	3.62	5.14	4.70	5.70
Exp 2	3.67	5.75	4.77	4.44	3.35
Macrophages					
Exp 1	2.96	4.02	5.60	5.26	5.65
Exp 2	3.67	5.20	6.39	6.20	6.81
TBARS					
Trophoblast					
Exp 1	1.26	3.13	3.11	3.14	4.66
Macrophages					
Exp 1	1.26	2.91	5.80	3.80	5.00

*Experiment 1 conducted for 3 hr; Experiment 2 for 6 hr.

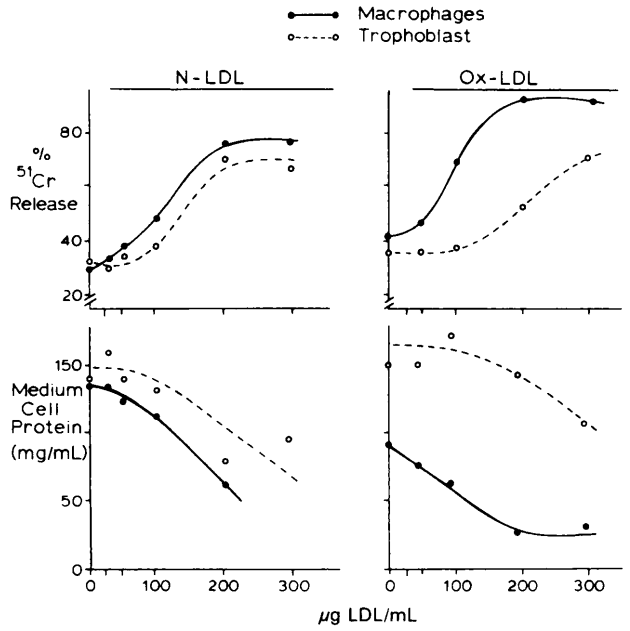

Figure 1. Effects of native-LDL and Ox-LDL on placental cell cytotoxicity. In this representative experiment, placental macrophages and trophoblast were incubated in HAM'S F-10 medium containing increasing concentrations of native-LDL or Ox-LDL. After 20 hr, ^{51}Cr release and cell protein content were determined. Both LDL and Ox-LDL increased ^{51}Cr release and decreased cell protein content in a concentration-dependent manner.

Table II. Cytotoxic Effects of Native and Oxidized LDL on Placental Macrophages and Trophoblast

	% ^{51}Cr release	
A: Native LDL:	(–)	(+)[a]
(*n*)	(6)	(6)
Macrophages	39.1 ± 1.7[b]	79.3 ± 2.2[d]
Trophoblast	36.2 ± 2.3	65.9 ± 4.2[d]
Macrophage vs trophoblast: *P*	> 0.30	< 0.02
B: Oxidized LDL:	(–)	(+)[a]
(*n*)	(7)	(7)
Macrophages	39.1 ± 1.7	67.8 ± 4.9[d]
Trophoblast	35.6 ± 1.3	48.1 ± 5.4[c]
Macrophage vs trophoblast: *P*	< 0.02	< 0.001

[a] 200 μg LDL protein added/ml medium; (*n*) refers to the number of experiments.
[b] Mean ± SE.
[c] $P < 0.05$ vs control.
[d] $P < 0.001$ vs control.

the Ox-LDL tended to be lower than with native LDL, probably reflecting a slower rate of further LDL oxidation in the already oxidized LDL.

To obtain further evidence that ongoing LDL oxidation is linked to placental cell injury, we perturbed the concentrations of divalent metal ions in the medium in various ways. First, EDTA was added in increasing concentrations (0.001–0.1 m*M*). As shown in Figure 2, increasing concentrations of EDTA decreased ^{51}Cr release and increased cell-protein content. Electrophoretic mobility of LDL was similarly diminished by increasing concentrations of EDTA (Fig. 3).

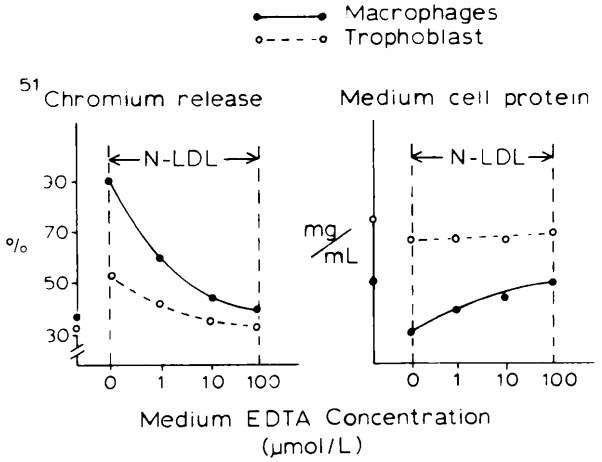

Figure 2. Inhibition of native-LDL mediated cytotoxicity by addition of EDTA. In this representative experiment, macrophages and trophoblasts were incubated in HAM'S F-10 medium containing LDL, 200 μg of LDL protein/ml medium, 5 μ*M* $CuCl_2$, and increasing concentrations of EDTA (0.001 to 0.1 m*M*). After 20 hr, ^{51}Cr release and cell protein content were determined as described in the methods. Symbols on the Y axis refer to ^{51}Cr release from trophoblast or macrophages incubated in the absence of LDL. In both cell populations, addition of LDL increased ^{51}Cr release and decreased cell protein content. Increasing concentrations of EDTA decreased ^{51}Cr release and increased cell protein content in a dose dependent manner.

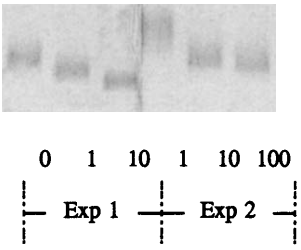

Figure 3. Inhibition of LDL electrophoretic mobility with increasing concentration of EDTA in the presence of 5 μ*M* $CuCl_2$ in macrophage culture. Macrophages were incubated in HAM'S F-10 medium containing native LDL as described in Figure 2. LDL electrophoretic mobility was determined on agarose gels after 20 hr incubation of LDL with $CuCl_2$ and increasing concentrations of EDTA. Increasing concentrations of EDTA decreased LDL electrophoretic mobility.

Secondly, the transition metal ion concentration was increased by adding $CuCl_2$ (5 μ*M*) to placental cell cultures in HAM's F-10 medium containing 100 μg LDL protein/ml. An increase in ^{51}Cr release was observed with the addition of Cu^{++} and LDL to both macrophages and trophoblast but the macrophages were again more sensitive to cytotoxicity stress than the trophoblast ($P < 0.001$) (Table III). The addition of LDL was associated with an increase in LDL electrophoretic mobility (Fig. 4). Addition of $CuCl_2$ to cell cultures in the absence of LDL had no effect on percentage of ^{51}Cr released (Table III). In contrast to the prooxidant effects of added Cu^{++} ion, the addition of lipoprotein-deficient serum (LPDS), which contains albumen and serum antioxidants, reduced LDL oxidation as demonstrated by diminished LDL electrophoretic mobility (Fig. 4) (see also below). These experiments further indicate the sensitivity of ongoing LDL oxidation to transition metal ion

Table III. Cytotoxic Effects of Copper Chloride in the Presence of Native-LDL

LDL 5 μM $CuCl_2$	% ^{51}Cr release			
	0		100 μg/ml	
	(−)	(+)	(−)	(+)
Macrophages	(7) 45.6 ± 2.2	(7) 48.6 ± 1.9	(24) 66.0 ± 2.7[b]	(24) 84.8 ± 2.0[a,c]
Trophoblast	(10) 32.6 ± 1.2	(8) 32.9 ± 1.1	(28) 34.2 ± 1.6	(28) 50.1 ± 2.5[a,c]
Macrophages vs trophoblast: *P*	< 0.001	< 0.001	< 0.001	< 0.001

Note. Macrophages and trophoblast were incubated in HAM's F-10 medium containing LDL (100 μg of LDL protein/ml of medium) and copper chloride to a final concentration of 5 μ*M*. Percentage of ^{51}Cr release indicates level of cytotoxicity. Values are mean ± SE. ( ): Denotes the number of experiments performed.
[a]: Effect of $CuCl_2$ versus none in the medium, $P < 0.001$.
[b,c]: LDL-containing versus LDL-free medium, $^bP < 0.01$, $^cP < 0.001$.

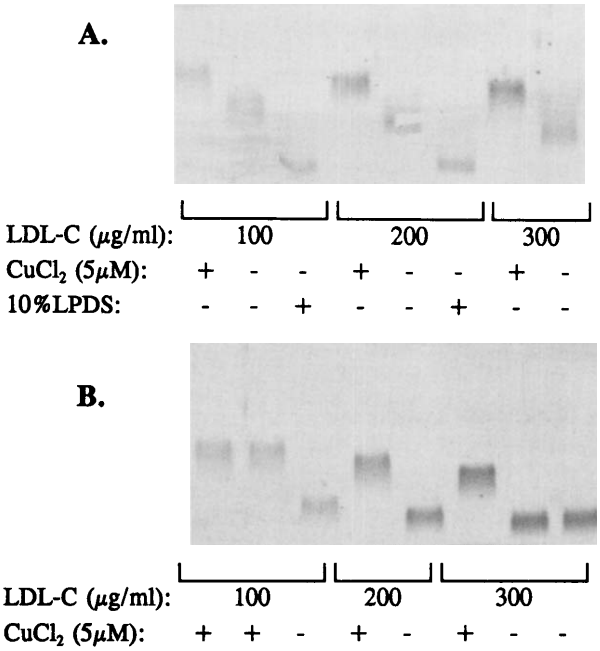

Figure 4. Effect of adding $CuCl_2$ on LDL electrophoretic mobility. LDL concentrations of 0, 100, 200, or 300 μg protein/ml medium were incubated with placental macrophages in HAM'S F-10 medium. Five μ*M* $CuCl_2$ was added in some wells. Then 10% (v:v) lipoprotein deficient serum (LPDS) was added to other wells. $CuCl_2$ accelerated and LPDS inhibited LDL oxidation as shown by increased and decreased LDL electrophoretic mobility, respectively. In general, mobility is less when LDL concentrations are greater. Similar results were found in trophoblasts incubated under the same conditions (data not shown). Panel B depicts a second experiment incubating increasing concentrations of LDL in HAM'S F-10 medium, ± 5 μ*M* $CuCl_2$ with placental macrophages. $CuCl_2$ again accelerated LDL oxidation as shown by an increased LDL electrophoretic mobility.

concentrations and the association of LDL oxidation with placental cell cytotoxicity.

Thirdly, placental macrophages and trophoblast were incubated with 200 μg LDL protein in either HAM's F-10 or MEM medium, the latter containing no transition metal ion. In macrophage culture, the ^{51}Cr release was significantly greater in the presence of LDL in HAM's F-10 medium compared to MEM medium ($P < 0.01$) (Table IV) where no increase in cytotoxicity was observed. In these experiments, trophoblast was little affected by the addition of LDL in either medium and was significantly less sensitive to oxidative stress than the macrophages ($P < 0.001$ in both media). Lipid peroxide formation in three experiments (Table V) and LDL electrophoretic mobility in two experiments (Fig. 5) were also consistently greater in cultures of placental macrophages and trophoblast incubated in HAM's F-10 medium compared to MEM medium.

Table IV. Effects of Ham's F-10 vs MEM Medium on LDL Induced Cytotoxicity

	% ^{51}Cr release		
	Macrophages	Trophoblast	Macrophages vs trophoblast *P*
Ham's F-10			
LDL:			
(−)	(12) 38.5 ± 1.4	(7) 38.0 ± 1.1	ns
(+)	(24) 51.0 ± 2.6	(11) 39.6 ± 0.6	< 0.001
P	< 0.01	ns	
MEM:			
LDL:			
(−)	(11) 40.2 ± 2.0	(7) 37.1 ± 3.6	ns
(+)	(23) 45.6 ± 2.2	(16) 34.9 ± 2.8	< 0.001
P	ns	ns	

Note. Values are mean ± SE. Cells were incubated in 200 μg/ml LDL protein.

The antioxidant BHT was also employed to test the association of LDL oxidation and cytotoxicity in cultured placental cells. BHT is a reactive oxygen species scavenger that interrupts the self-perpetuating cycle of oxygen-free

Table V. Effect of Incubating Native-LDL in HAM's F-10 vs MEM Media on Lipid Peroxide Formation

Media	nmoles/100 μl medium			
	Macrophages		Trophoblasts	
	Ham's F-10	MEM	Ham's F-10	MEM
Exp 1	0.9 ± 0.04	ND	1.6 ± 0.01	ND
Exp 2	8.9 ± 0.09	ND	11.2 ± 0.10	ND
Exp 3	2.1 ± 0.20	1.2 ± 0.1	3.0 ± 0.70	0.7 ± 0.3

Note. Mean ± SE of replicate observations in each experiment; ND, not detectable; LDL present at a concentration of 200 μg/ml.

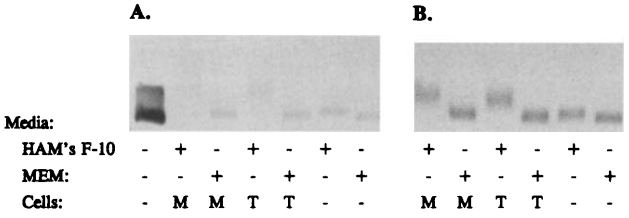

Figure 5. Electrophoretic mobility of LDL after incubating in HAM's F-10 and MEM media containing and lacking transition metal ion, respectively, in the presence of placental cells. Macrophages and trophoblast were incubated in the two media using two LDL donors, panel A and panel B. LDL was incubated at a concentration of 200 μg protein/ml for 200 hr. In both panels, Lanes 1 and 2 are from macrophage (M) cultures, Lanes 3 and 4 are from trophoblast (T) cultures, and Lanes 5 and 6 are from blank wells. LDL electrophoretic mobility is consistently greater in incubation from HAM's F-10 than MEM. Native nonincubated LDL is shown in the extreme left lane of Panel A.

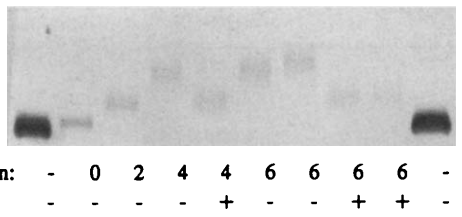

Figure 6. Effects of BHT on LDL electrophoretic mobility. Macrophages were incubated with LDL (200 μg protein/ml medium) in HAM'S F-10 containing 5 μ*M* $CuCl_2$. Then 2 hr after the beginning of the experiment, BHT was added to a final concentration of 0.1 m*M*. Electrophoretic mobility was determined after 0, 2, 4, and 6 hours on agarose gel. The first and last lanes represent the mobility of unincubated LDL.

radical generation and LDL oxidation and blunts LDL oxidation and cell toxicity. Macrophages and trophoblast were incubated in HAM's F-10 medium in the presence of Ox-LDL (200 μg/ml) and increasing concentrations of BHT. As shown in Table VI, ^{51}Cr release was reduced in macrophage cultures at all concentrations of BHT. Trophoblast incubated with Ox-LDL showed no significant increase in ^{51}Cr release, and BHT showed little benefit (Table VI). However, LDL electrophoretic mobility (Fig. 6) and lipid peroxide formation (Table VII) were consistently reduced by BHT in three separate experiments of trophoblast and macrophages. These results are in keeping with our previous reports of inhibition of LDL-mediated cytotoxicity in trophoblast and macrophages by increasing concentrations of 17β-estradiol (15), owing to the hydroxyphenol structure of the A ring of that molecule. Also, consistent with an antioxidant effect is the diminished cytotoxicity of 200 μg LDL protein in macrophages with addition of lipoprotein deficient serum (LPDS): percentage of ^{51}Cr released was (8) 81.0% ± 2.3% *vs* (7) 55.6% ± 62% with mean ± SE addition of 10% LPDS ($P < 0.001$). In trophoblast, the percentage of ^{51}Cr released, was 65.5% ± 5.3% in the absence and 48.9% ± 5.7% in the presence of LPDS ($P < 0.05$).

Discussion

Pregnancy is a condition of enhanced oxidative stress as judged from increased concentrations of lipid peroxides present in plasma of normal pregnant women and women with toxemia in pregnancy (5–11) and conjugated dienes, a measure of lipid oxidation (7, 8). Lipid peroxides have been found in placentas of toxemic pregnancies by Walsh *et al.* (12) and Wang *et al.* (28). We (8) and others (9) have observed increased susceptibility of LDL to oxidation in the presence of copper ion in normal and diabetic pregnancy as gestation proceeds. Most recently, Branch and associates have found antibodies to oxidized LDL in toxemic pregnancy (14). These data provide considerable evidence for increased oxidative stress on lipids and lipoproteins in pregnancy.

Enhanced LDL oxidation may occur in pregnancy because maternal blood is in direct contact with placental trophoblast that lacks an endothelial covering of the placental villae. This slow-flow, low-pressure circulation allows prolonged exposure of circulating lipoproteins to the trophoblast surface (29) as depicted in Figure 7. One placental defense mechanism against modified lipoproteins in the placental circulation is scavenger receptors of trophoblast and macrophages (4, 30, 31). Should the rate of LDL oxidation exceed the capacity of placental defenses, the cytotoxic potential of oxidized LDL could cause direct damage to the placental structure. Therefore, we wished to confirm and extend our previous observations that placental cells oxidize LDL (15), and LDL oxidation is linked to placental cell damage (15).

When increasing numbers of placental cells were incubated with native LDL, LDL oxidation was accelerated by placental cells in the form of lipid peroxides and TBARS formation (Table I). These results confirm our recently published observations that LDL oxidation was increased as a function of time of exposure to LDL in placental cell culture (15).

Knowing that oxidized LDL causes cytotoxicity to other cell systems (31–34), placental cells were exposed to copper-oxidized as well as native LDL. Native LDL and

Table VI. BHT Inhibition of Ox-LDL Induced Cytotoxicity

	% ^{51}Cr release				
Ox-LDL (μg/ml)	0	200	200	200	200
BHT (μ*M*)	0	0	1	10	100
Macrophages	49 ± 3[a]	72 ± 6[b]	54 ± 6	54 ± 7	55 ± 4
Trophoblast	33 ± 1	35 ± 2	39 ± 2	35 ± 2	35 ± 1

[a] Mean ± SE of three experiments; [b] $P < 0.001$ vs control.

Table VII. Inhibition of Lipid Peroxide Formation by BHT in Placental Cell Cultures in the Presence of Ox-LDL

BHT (μ*M*)	nmoles/100 μl of medium			
	0	1	10	100
Macrophages				
Exp 1	0.6 ± 0.1	0	0	0
2	3.9 ± 0.1	2.1 ± 0.3	0.8 ± 0.4	2.2 ± 0.1
3	9.6 ± 0.3	7.0 ± 1.2	7.8 ± 2.1	3.2 ± 0.1
Trophoblast				
Exp 1	2.4 ± 1.0	1.3 ± 0.1	0.6 ± 0.1	1.4 ± 0.3
2	4.5 ± 0.5	2.5 ± 0.4	1.6 ± 0.1	1.6 ± 0.2
3	14.5	13.6 ± 1.6	14.0 ± 0.3	7.6 ± 0.0

Notes. Values are mean ± SE of 2–3 replicate observations in each experiment. Ox-LDL present at a concentration of 200 μg protein/ml.

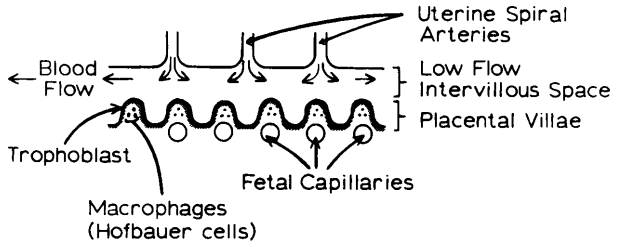

Figure 7. Schematic representation of the low-pressure, slow flow of maternal blood from the uterine spinal arteries to the intervillous space, bathing the placental villae. Trophoblast covers the surface of the villae while placental macrophages (Hofbauer cells) occupy the interior of the villae.

oxidized LDL both caused cytotoxicity as demonstrated by increased ^{51}Cr release and cellular-protein loss. Native LDL was as cytotoxic as the previously copper-oxidized LDL, if not more so (Fig. 1, Table II), suggesting that it is the ongoing oxidation of LDL that mediates placental cell cytotoxicity rather than a toxin inherent in the oxidized LDL.

The link between ongoing LDL oxidation and placental-cell toxicity was shown further by manipulating transition metal ion concentrations in various ways or by adding the free radical scavenger BHT or lipoprotein-deficient serum containing albumen and antioxidants. Increases in LDL oxidation were associated with an increase in cytotoxicity. When the transition metal ion chelator EDTA was added, LDL oxidation and placental-cell injury decreased (Figs. 2 and 3) with the greater effect observed in macrophages, which seem more susceptible to cytotoxicity than trophoblast. A similar result was seen when cells were incubated in media lacking transition metal ions (MEM) compared to media containing transition metal ions (HAM's F-10). Again, in this system, macrophages showed the greater susceptibility to cytotoxicity compared to trophoblast (Table IV), despite similar increases in indices of oxidation in both placental cell types in the presence of transition metal ions (Table V and Fig. 5). Likewise, addition of $CuCl_2$ increased LDL oxidation (Fig. 4) and placental cell cytotoxicity (Table III).

The final association of LDL oxidation and cytotoxicity was demonstrated by addition of BHT, which attenuated LDL oxidation and cell cytotoxicity in macrophages, where the cytotoxic effect was seen (Tables VI and VII, Fig. 6). A similar inhibition of LDL oxidation and cytotoxicity was seen when lipoprotein-deficient serum was added to cultures of both macrophages and trophoblast. These results indicate that the ongoing process of LDL oxidation is responsible for the cytotoxic effect on placental cells and that this process can be accelerated or diminished as LDL oxidation is accelerated or diminished just as oxidized LDL is linked to the process of arterial wall injury and atherogenesis (32–38). These results confirm the linear association of LDL oxidation and trophoblast and placental macrophage cytotoxicity where additions of 17β-estradiol inhibit and progesterone or testosterone accelerate this process (15).

Clinical observations point to a link between oxidative stress in pregnancy and placental dysfunction. Oxidative stress is enhanced in pregnancies complicated by insulin-dependent diabetes (8, 9), toxemia (5, 6, 10–13) and cigarette smoking (39). In many of these cases a structurally abnormal, small or poorly functioning placenta is found (40–45). In some diabetic placentas, obliterative endarteritis of fetal stem cells and villous fibrinoid necrosis are observed (42). The placental cell toxicity observed with LDL oxidation in the present experiments provides a plausible mechanism for the observed clinical associations.

The apparent greater sensitivity of macrophages to oxidative stress compared to trophoblast deserves comment. The resistance of trophoblast to LDL oxidation may be due to the larger cell mass of syncytiotrophoblast than macrophages in culture, the 3-fold greater density of scavenger receptors in trophoblast, which can remove acetylated and oxidized LDL from the circulation or other unknown factors such as durability in primary tissue culture (4). If the resistance of trophoblast to LDL oxidation induced cytotoxicity exists *in vivo,* this adaptation may serve a survival and reproductive advantage as a barrier to oxidative stress upon the fetus. The importance of this defense is underscored by recent studies of Ericson *et al.* and Viana *et al.,* who have shown that oxidative stress and antioxidant defenses in the embryo are fundamental to the causation of teratogenesis (46, 47). Nonetheless, if this defense is overcome by a strong oxidant stress, the trophoblast is susceptible to oxidative damage and cytotoxicity as demonstrated in the present experiments.

In summary, these studies confirm that placental cells accelerate LDL oxidation. In turn, LDL oxidation is toxic to cultured placental macrophages and to a lesser extent trophoblast. LDL oxidation by placental cell cultures is stimulated by metal ions and is inhibited by antioxidant and by lipoprotein-free serum. If this oxidative process occurs *in vivo,* placental injury may result, and fetal growth may be impaired. This model system should stimulate future *in vivo* studies to determine if placental damage occurs in pregnancies subjected to oxidative stress.

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