Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid

Diane W. Morel,*, James R. Hessler,* and Guy M. Chisolm*,**,[1]**

Department of Cardiovascular Research, Division of Research, Cleveland Clinic Foundation,* and Department of Biomedical Engineering, Case Western Reserve University,** Cleveland, OH 44106

Abstract Low density lipoprotein (LDL) has been reported to be injurious or toxic to cells in vitro. This injurious effect is, in some instances, due to oxidation of the lipid moiety of the lipoprotein. The objectives of this study were to determine if the oxidation rendering the lipoprotein toxic to human skin fibroblasts occurred by free radical mechanisms, and if so, which of the common free radical oxygen species were involved. The selective free radical blockers or scavengers employed included superoxide dismutase for superoxide, catalase for hydrogen peroxide, dimethylfuran for singlet molecular oxygen, and mannitol for hydroxyl radical. The presence during lipoprotein preparation of general free radical scavengers (vitamin E, butylated hydroxytoluene) or the divalent cation chelator ethylenediamine tetraacetic acid prevented the formation of cytotoxic low density lipoprotein, while the simultaneous presence of superoxide dismutase and catalase partially inhibited its formation. The results indicate that superoxide and/or hydrogen peroxide are involved in the formation of the toxic LDL lipid. The toxic action of oxidized LDL could not be prevented by inclusion of antioxidants in the culture medium, indicating that an oxidized lipid was responsible for cell injury rather than free radicals generated in culture by the action of oxidized LDL. Three separate assays for cell injury (enumeration of attached cells, cell loss of lactate dehydrogenase into the culture medium, and trypan blue uptake) indicated a sequence of events in which the fibroblasts are injured, die, and then detach.—**Morel, D. W., J. R. Hessler, and G. M. Chisolm.** Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J. Lipid Res.* 1983. **24:** 1070–1076.

Supplementary key words cell injury • cultured skin fibroblasts • free radical scavengers

Low density lipoprotein (LDL) is injurious or toxic under certain conditions to human (1–3), bovine (4), and porcine (5) vascular cells, and to human fibroblasts (6–9) in culture. LDL-induced cell injury has been monitored by microscopic observation (1–9), enhanced release of cell-associated ^{51}Cr into the culture medium (2, 6), decreased cellular uptake of [^{3}H]adenine (5), and decreased numbers of adherent cells in the culture dish (1, 7, 8). It is unclear from the studies reporting cytotoxicity whether or not cell detachment precedes cell death.

Recent reports indicate that cytotoxicity induced by LDL (5, 7–10) and very low density lipoprotein (8) can result from lipoprotein oxidation. Oxidation in these studies is indicated by the relative amount of malondialdehyde equivalents or thiobarbituric acid-reacting substances (TBARS) residing on the lipoprotein. The presence of the antioxidant glutathione or the metal ion chelator ethylenediamine tetraacetic acid (EDTA) throughout the LDL isolation from normal plasma or serum can prevent the formation of cytotoxic LDL (7–9). Both the TBARS and the cytotoxic agent(s) are associated with the lipid-extractable portion of the LDL molecule (8).

It is well known that lipid peroxidation can occur through the interactions of free radicals and lipids. Lipid peroxides and free radicals are elevated in certain pathological conditions. If the lipid peroxide-associated cytotoxicity observed in vitro has an in vivo analog, an important link may exist between free radical concentrations (or lipid peroxides) and tissue damage accompanying various diseases. Reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet molecular oxygen are generated in vivo under various conditions. These include ethanol-induced liver injury (11), chlorinated hydrocarbon hepatotoxicity (12), and exposure to ionizing radiation (13). Complement-activated neutrophils can also generate reactive oxygen species (14, 15). In vitro studies indicate that oxygen radicals are released by complement-stimulated granulocytes (16) and phagocytosing polymorphonuclear leukocytes (17). These oxygen species can damage cells directly by interacting with structural components of membranes and membrane enzymes or by initiating

[1] To whom reprint requests or inquiries should be addressed at Wickenden 507, Dept. of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106.

Abbreviations: BHT, butylated hydroxytoluene; HBSS, Hank's balanced salt solution; CAT, catalase; EDTA, ethylenediamine tetraacetic acid; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reacting substances.

lipid peroxidation reactions (18). Lipid peroxidation can also be initiated by the reaction of oxygen and polyunsaturated lipids to form free radical intermediates (19). The peroxy radicals formed can interact with and damage membranes and subcellular organelles (20). The role of various free radicals in lipid peroxidation that leads to lipoprotein cytotoxicity has not been reported.

In the present studies we show that the oxidation of LDL which renders it cytotoxic occurs by free radical mechanisms involving superoxide and/or hydrogen peroxide. Furthermore, cytotoxicity is not the result of direct interaction between these oxygen free radicals and the cells but rather the interaction between the cells and a lipid peroxide. Our results also show that exposure of fibroblasts to this LDL-borne lipid peroxide results in cell death with subsequent detachment from the culture dish.

METHODS

Cell preparation

Human dermal fibroblasts were obtained from post-circumcision neonatal foreskins. After the epidermal layers were removed by careful dissection, portions of the underlying dermis were cut into small (1 mm^3) explants and placed in 25-cm^2 flasks (Falcon). Specimens were incubated with Morgan's M-199 medium (GIBCO) supplemented with 0.3 mg/l glutamine, 10 mM HEPES buffer, and 15% fetal bovine serum (MA Bioproducts) in a humidified 5% CO_2 and air environment. Culture medium was changed twice weekly. By 3–4 weeks, when sufficient peripheral growth had occurred, cells were subcultured into larger (75 cm^2) flasks using 0.1% trypsin in Hank's balanced salt solution (HBSS, GIBCO) without Ca^{2+} and Mg^{2+}. All experiments were performed with cells in passage 2 through 8. Cell seeding densities of $1.7–2.4 \times 10^5$ cells per 35 mm culture dish (GIBCO) were routinely used for experimentation. Dermal specimens from several donors were used in the course of these studies.

Lipoprotein preparation

Lipoproteins were prepared from freshly drawn (<24 hr) citrated human plasma from normolipemic individuals (plasma from many different donors was used in the studies). Plasma solvent density was adjusted to 1.019 g/ml with a high density salt solution (containing NaCl, KBr, and EDTA) as described by Hatch and Lees (21). After centrifugation for 22 hr at 40,000 rpm, 7°C in a Beckman L5-75 ultracentrifuge using a 50.3 rotor, the supernatants were removed. The pooled infranatant was adjusted to density 1.060 g/ml, recentrifuged as before, and LDL_2 was collected. LDL_2 (d 1.019–1.060 g/ml) was used for experimentation and is referred to as LDL throughout this paper. Lipoprotein deficient serum (LPDS, d > 1.25 g/ml) was prepared from pooled whole human serum.

Preparations were dialyzed at 4°C for 48–62 hr against four to six changes of at least 50 volumes of dialysate (0.15 M NaCl), adjusted to pH 7.2–8.0. For the experiments in which dialysate composition was varied, the pH, the number of changes, and the time of dialysis were the same. Butylated hydroxytoluene (BHT), vitamin E acetate, and β-carotene (all from Sigma) were dissolved in absolute ethanol and added 1:100 (v/v) to the isotonic saline dialysate to achieve concentrations of 20 μM, 22 μM, and 18.6 μM, respectively. Other free radical scavengers were added to the isotonic saline dialysate directly to achieve final concentrations of 1.3 mM EDTA, 20 mM mannitol (Sigma), 20 mM 1,4-diazobicyclo[2.2.2]octane (Aldrich), and 9.3 mM 2,5-dimethylfuran (Aldrich). Superoxide dismutase (SOD, Sigma) and catalase (CAT, Sigma) were added to the LDL sample (instead of to the dialysate since the molecular weights are greater than 12,000) to achieve final concentrations of 5.9 μM and 0.83 μM, respectively, and dialyzed against isotonic saline.

After filtration sterilization (0.45 μM, Millipore) of the LDL or LPDS, cholesterol and triglyceride concentrations were determined by the Lipid and Lipoprotein Laboratories of the Cleveland Clinic Foundation (automated analyses standardized and certified by the Center for Disease Control, Atlanta, GA). Protein concentrations were determined by the method of Lowry et al. (22).

Human lipoprotein preparations and LPDS were analyzed by agarose gel electrophoresis and immunoelectrophoresis (with anti-sera to human whole serum, albumin, alpha- and beta-lipoproteins). LPDS was found to be virtually free of cholesterol and triglyceride.

Assay for thiobarbituric acid-reacting substances (TBARS)

Malondialdehyde content of lipoprotein samples was determined spectrophotometrically using a modification of the assay described by Schuh et al. (23). Specifically, 1 ml of 20% trichloroacetic acid was added to 100 μl of a lipoprotein preparation of known cholesterol, protein, and triglyceride concentrations. Following this precipitation, 1 ml of 1% thiobarbituric acid was added, the mixture was incubated at 95°C for 45 min, cooled, and subsequently centrifuged at 1000 *g* for 20 min. The absorbance of the supernatant was determined immediately using a Gilford 250 spectrophotometer, at wavelength 532 nm. Freshly diluted malondialdehyde bis(dimethyl acetal)(1,1,3,3-tetramethoxypropane, Aldrich) was used as a standard.

For the experiment in which the pH of the thiobarbituric acid assay for malondialdehyde was altered, a modification of the method outlined by Ohkawa, Ohishi, and Yagi (24) was used. To 100 μl of an LDL sample of known cholesterol concentration were added 0.2 ml of 8.1% sodium dodecylsulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 4.0 with NaOH, and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. The mixture was made up to 4.0 ml with 0.7 ml of distilled water. After each addition the reaction mixture was thoroughly mixed. It was heated at 95°C for 60 min, then cooled with tap water. The absorbance at 532 nm was measured immediately for freshly diluted standards or samples.

In vitro incubations

Fibroblasts were trypsinized and plated into 35-mm culture dishes in M-199 with 15% fetal calf serum 24–36 hr prior to the start of experimentation. After this period, all cultures were rinsed three times with HBSS, and to each was added medium M-199 containing LPDS (4.0 mg protein/ml of medium) supplemented with the lipoprotein or its dialysate (as control) for 3 days of study.

In all trials the medium composition by volume was 80% M-199, 10% LPDS, and 10% LDL (or a control with the same composition as the final LDL solution, but without LDL). LDL preparations routinely had cholesterol concentrations between 400 and 700 mg/dl and were diluted with their respective dialysates before use so that the desired final concentration of each fraction could be attained in the culture medium with the 10% medium dilution.

Population changes (growth or toxicity) were assessed qualitatively by daily inspection of the culture dishes using an inverted stage microscope, and quantified at termination by counting suspended cells with a ZBI Coulter Counter (100 μm aperture), as previously described (2). Our index of cytotoxicity was the number of cells remaining attached at termination in the experimental groups expressed as a percent of the cells remaining attached at termination in the control groups (±SEM). Typically the cells doubled or tripled in the control groups during the course (66 hr) of an experiment. Final cell counts in an experimental group in which cytotoxicity was observed were less than the cell counts at the start of the experiment.

In an experiment to determine whether toxicity preceded cell detachment, viability of cells still attached to the dish was determined by trypan blue dye exclusion (25). At each time point the medium was removed from the dishes and the dishes were rinsed twice with HBSS. To each dish 0.9 ml of HBSS and 0.1 ml of 0.4% solution of trypan blue in isotonic saline were added. After a 5-min incubation the dye was removed and each dish was washed twice with HBSS. The number of cells taking up and excluding the dye were counted under the microscope in three "peripheral" and three "center" fields of each dish. The average percent of attached cells in the field stained with trypan blue was recorded.

Lactate dehydrogenase determination was performed with a kit (Sigma) according to the method of Wroblewski and LaDue (26). To the premeasured vial of nicotinamide adenine dinucleotide was added 2.85 ml of 0.1 M potassium phosphate buffer, pH 7.5, and 0.1 ml of medium. These were mixed well and left at 25°C for 20 min. Then 0.1 ml of sodium pyruvate solution was added, and the vial was capped and mixed by inversion. After transferring the mixture to a cuvet of 1-cm light path, the absorbance at 340 nm was read at 30-sec intervals for 6 min at constant temperature using water as a reference. Lactate dehydrogenase activity, expressed as units per ml of medium, was determined by multiplying the change in absorbance per minute by a factor of 20,000 and a temperature correction factor for compartment temperatures other than 25°C.

All experiments were performed using triplicate dishes in each group. Statistical significance was calculated using the two-tailed *t*-test for unpaired data.

RESULTS

Comparison between cell enumeration as an index of cytotoxicity and two alternate indices is displayed in **Fig. 1** and **Table 1.** These alternate measures take advantage of the facts that LDH, a cytosolic enzyme, is released from a cell when it is injured, and that trypan blue dye is taken up by dead and dying cells but excluded by viable cells. Fig. 1 shows that longer cell exposure to LDL resulted in an increase in the amount of lactate dehydrogenase released into the medium. This elevation of lactate dehydrogenase was concomitant with a decrease in the number of cells attached to the dish, an indication that detachment did not significantly precede cell death. As seen in Table 1, with increasing time of LDL exposure, the percentage of cells that were still attached to the dish but stained with trypan blue increased. This increase also coincided with the decrease in the number of cells remaining attached to the dish (percent of control). Despite efforts to obtain a uniform distribution of cells seeded in a culture dish, the center of the dishes had a more dense population of cells than the periphery. The delayed increase in staining with trypan blue in the center areas correlated with microscopic observations of less rapid cell detachment in the center than at the periphery. In other experiments, all detached cells recovered from the me-

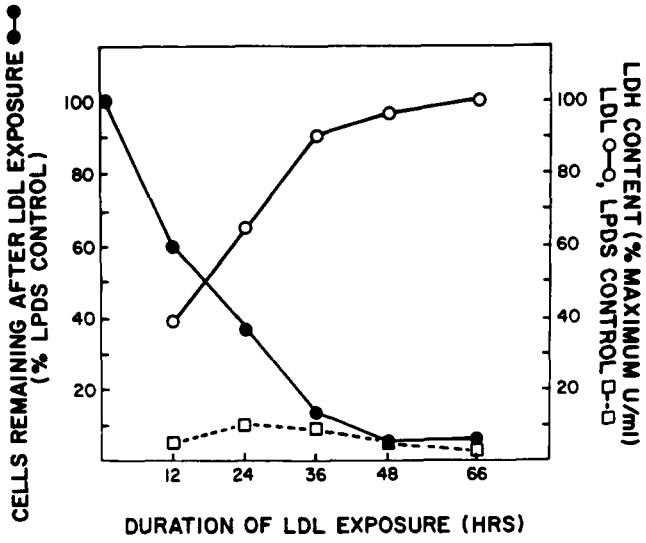

Fig. 1. The effect of the duration of cell exposure to oxidized LDL on both the survival of cultured human skin fibroblasts and the amount of lactate dehydrogenase (LDH) expressed as percent of the maximum (100% = 46 Wroblewski units per ml of medium). LDL concentration was 225 μg LDL-chol/ml medium. LDH is the average of duplicates; relative cell survival is the average of triplicates as given in Table 1, last column. Average number of cells in LPDS control at 66 hr was 5.8×10^5 cells/dish; average number of cells at start of 66-hr period was 1.64×10^5 cells/dish.

dium and exposed to trypan blue took up the dye. Based on these results, it appears that LDL exposure caused cell death before the cells detached from the culture dish. Cells attached to the dish at termination represented viable cells plus those injured or dead, whereas cells that had detached represented only dead cells. Thus, while a decrease in the number of cells remaining attached to the dish after a given period of LDL exposure definitely reflects cell death, it is not the earliest indication of reduced viability.

We previously observed that the presence of glutathione during LDL dialysis prevented the formation of toxic LDL, and we attributed this effect to antioxidation (8). **Fig. 2** indicates that blocking all free radicals with BHT or vitamin E prevents the production of toxic LDL. Divalent copper and iron ions can act as catalysts for oxidation and free radical generation. Trace amounts of these ions may be responsible for free radical production during dialysis because EDTA, a divalent ion chelator, also completely prevented the formation of toxic LDL (Fig. 2).

Neither superoxide dismutase (SOD), an enzyme that inactivates superoxide anion (16–18), nor catalase (CAT), an enzyme that inactivates hydrogen peroxide (16–18) were able by themselves to prevent the formation of toxic LDL when added separately to the LDL during dialysis (Fig. 2A). However, SOD and CAT added simultaneously to LDL partially inhibited formation of toxic LDL. Increasing the concentrations of SOD and CAT in the LDL preparation (10-fold) or increasing the frequency of adding fresh enzyme to the LDL during dialysis did not change the extent of protection (data not shown). Mannitol, a scavenger of hydroxyl radical (17), did not prevent toxic LDL formation, nor did singlet molecular oxygen scavengers such as 1,4-diazobicyclo[2.2.2]octane (19), 2,5-dimethylfuran (19), and β-carotene (18).

In these experiments we used the thiobarbituric acid assay as an indicator of oxidation. In all tests we have performed using this assay, the detection of thiobarbituric acid-reacting substances (TBARS) in an LDL sample was indicative of cytotoxicity. For the experiment shown in Fig. 2A, the EDTA and BHT groups had no detectable TBARS and the other groups had varying amounts: SOD + CAT had 3.1 nmol MDA equivalents/mg LDL chol; SOD, 4.3; CAT, 4.4; mannitol, 5.1; and saline alone, 4.4. For those in Fig. 2B, the EDTA and vitamin E groups had no detectable TBARS, SOD + CAT had 2.1; β-carotene, 3.8; 2,5-dimethylfuran, 4.1; 1,4-diazobicyclo[2.2.2]octane, 4.2; and saline alone, 3.9.

There is a wide range of different assays which have been used to measure TBARS. One of the variables in these different assays is the pH at which the reaction is performed. Our usual assay employed trichloroacetic acid and was carried out at a pH less than one. Ohkawa et al. (24) reported that the pH of the reaction mixture determines the assay sensitivity to peroxides of various unsaturated fatty acids. At low pH the assay is relatively insensitive to linoleic acid hydroperoxide but is more sensitive to linolenic and arachidonic acid hydroperoxides. At a higher pH (3–4), the assay is equally sen-

TABLE 1. The effect of the duration of cell exposure to oxidized LDL on the survival of cultured human skin fibroblasts and the uptake of trypan blue dye by those cells still attached to the dish

Length of Exposure to Oxidized LDL	% of Attached Cells Stained with Trypan Blue (i.e., injured or dead)		Cells Remaining after LDL Exposure (% LPDS control)
	Center	Periphery	
hr			
12	4.9	28.9	59.3 ± 2.3
24	18.8	45.0	36.2 ± 2.6
36	26.9	56.4	14.0 ± 0.8
48	42.8	52.3	7.4 ± 0.4
66	58.5	58.5	8.2 ± 1.1

Cells from the center (more dense) and peripheral regions (less dense) of the culture dishes were separately quantified. LDL concentration was 225 μg LDL-chol/ml medium. The percentage of cells stained with trypan blue is the average of duplicates; cell survival is the average of triplicates ± SEM. Average number of cells in LPDS control at 66 hr was 5.8×10^5 cells/dish; average number of cells at start of 66-hr period was 1.64×10^5 cells/dish.

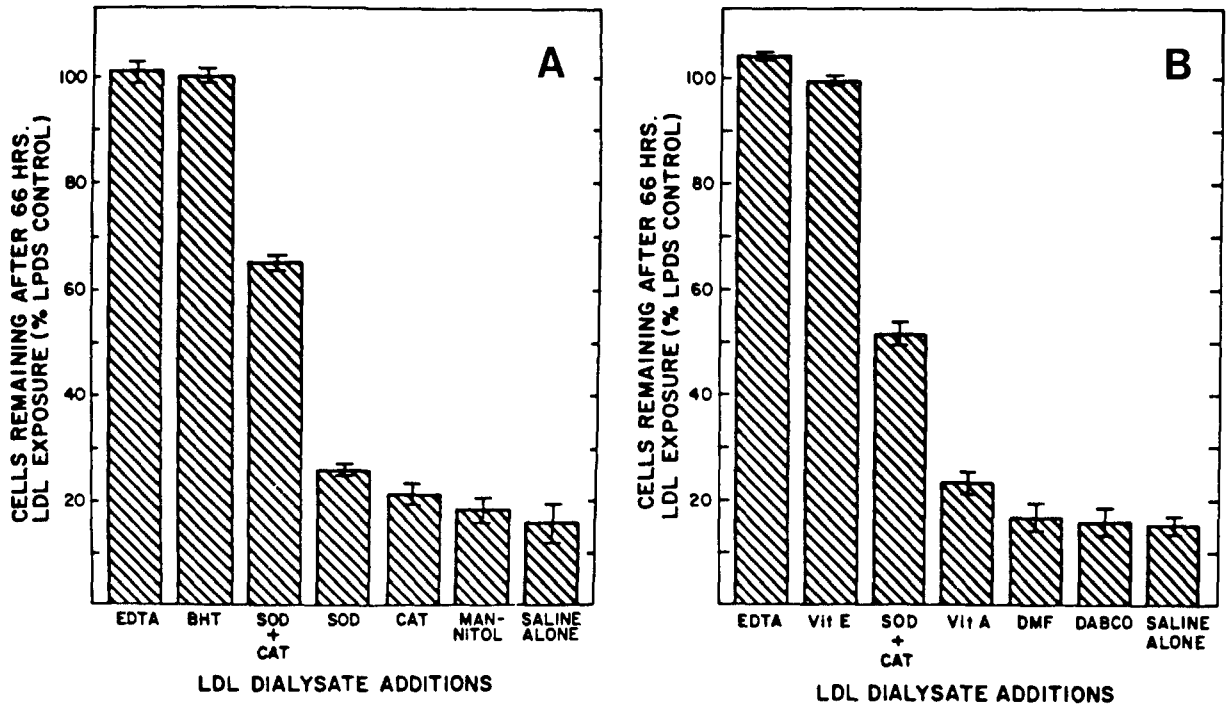

Fig. 2. The effect of various dialysate conditions (in the final step of preparing LDL prior to its addition to the culture medium) on the 66-hr survival of cultured human skin fibroblasts. LDL concentration was 250 μg LDL-chol/ml medium. In A, dialysate concentrations were 1.3 mM EDTA, 20 μM BHT, and 20 mM mannitol. In B, dialysate concentrations were 1.3 mM EDTA, 22 μM vitamin E acetate (Vit E), 18.6 μM β-carotene (Vit A), 9.3 mM 2,5 dimethylfuran (DMF), and 20 mM 1,4-diazobicyclo[2.2.2]octane (DABCO). In both A and B, SOD and CAT were added to the LDL in the dialysis bag to final concentrations of 5.9 μM and 0.83 μM, respectively. Cell survival is the average of triplicates ± SEM. Average number of cells in LPDS control at 66 hr varied from 3.0 to 3.4 × 10^5 cells/dish for the groups in A, and from 4.0 to 4.2 × 10^5 cells/dish for the groups in B. Average number of cells at start of the 66-hr period was 1.44 × 10^5 cells/dish for A and 1.66 × 10^5 cells/dish for B.

sitive to all three hydroperoxides. When we compared the ability of our assay (pH 1.0) and that at a higher pH (pH 3.5) to predict the toxicity of an LDL sample, we found that the assay at low pH was more effective. While dialysis against glutamine was shown by others to inhibit formation of toxic LDL while allowing oxidation (5), we observed that glutamine in the dialysate (4 mM) resulted in detectable TBARS formation in the LDL sample only when measured by the pH 3.5 assay (19.3 nmol MDA/mg LDL chol), but not by the pH 1 assay. We confirmed that the resulting LDL was not cytotoxic. The pH 3.5 assay, in general, yielded higher values of TBARS than the pH 1 assay.

After initiation of oxidation by dialysis without antioxidants, LDL continued to oxidize in storage as reported by Lee (27). Over a period of time this was visually monitored by a change in color from yellow-gold to increasingly pale yellow. This color change was accompanied by an increase in the TBARS content of the LDL. If BHT was added to a sample of oxidized LDL stored under nitrogen, the process was inhibited.

It is probable that further oxidation of LDL occurs while it is in the culture medium during the course of a normal 3-day experiment. In order to determine whether the propagation of the oxidative reaction in the culture dish was necessary to the resultant toxicity, antioxidants were added to cultures together with oxidized LDL. The results are displayed in **Table 2.** Whereas the presence of EDTA, BHT, or SOD and CAT during dialysis inhibited the formation of toxic LDL, their presence in culture with oxidized LDL did not inhibit toxicity. Thus, it is likely that an oxidized lipid and not a free radical or intermediate substance generated during propagation of peroxidation was responsible for LDL toxicity.

DISCUSSION

In this report we have verified that the adverse effect of oxidized LDL on cells in culture does in fact result in cell death. The increased release of lactate dehydrogenase into the medium and the increased uptake of trypan blue represent cell injury that corresponded with the decreased number of adherent cells. Thus, cell enumeration in this case is a reliable index of cell death.

The results of this study show that free radicals are involved in the LDL oxidation that renders LDL cytotoxic. The oxidation and the generation of toxic LDL

TABLE 2. Thiobarbituric acid-reacting substances and survival of human fibroblasts

Dialysate Conditions	Culture Conditions	TBARS (nmol MDA/ml medium)	Cells Remaining after 66 hr (% LPDS control)
+EDTA		0.0	97.5 ± 0.8
+BHT		0.0	95.9 ± 4.2
+SOD/CAT		0.387	63.5 ± 1.6
Saline only		0.675	32.7 ± 7.0
Saline only	+EDTA	0.675	34.0 ± 2.3
Saline only	+BHT	0.675	33.8 ± 9.3
Saline only	+SOD/CAT	0.675	33.1 ± 7.4

Thiobarbituric acid-reacting substances (TBARS, measured in malondialdehyde (MDA) equivalents) and survival of cultured human skin fibroblasts after 66 hr exposure to LDL plus antioxidant added either to the LDL during preparation or to the culture medium. LDL concentration was 225 μg LDL-chol/ml medium. Final medium concentrations of antioxidants were 65 μM EDTA, 1 μM BHT, 0.3 μM SOD, and 0.04 μM CAT. TBARS is the average of duplicates; cell survival is the average of triplicates ± SEM. Average number of cells in LPDS controls at 66 hr varied from 4.0 to 4.2 × 10^5 cells/dish for the various groups shown; average number of cells at start of 66-hr period was 1.66 × 10^5 cells/dish.

were completely prevented by the presence during LDL preparation of BHT or vitamin E, both general free radical scavengers. Further increases in TBARS of oxidized LDL were inhibited by the addition of BHT. Free radicals are not, however, the direct effectors of cell damage since the combined addition of BHT and oxidized LDL to the culture medium did not alleviate the cytotoxicity of oxidized LDL.

Specific free radical scavengers were employed as selective blockers of oxidation in an attempt to determine which free radicals are involved in LDL oxidation. Of these, only the combined addition of SOD and CAT during LDL preparation was effective as an inhibitor of LDL oxidation (9, 10). The fact that only partial protection from toxicity was achieved by the combined addition of SOD and CAT, whereas no protection was achieved by the addition of either alone (see Fig. 2A), may be explained by the reported inhibition of CAT by superoxide radical and the inhibition of SOD by hydrogen peroxide (28). When SOD or CAT alone are employed as scavengers, the presence of hydrogen peroxide or superoxide, respectively, can inhibit the scavenging ability of each enzyme. When SOD and CAT are present simultaneously during LDL preparation, each enzyme may improve the ability of the other to scavenge its target free radical by reducing the concentration of its inhibitor. In this way, SOD and CAT can act synergistically to effect the partial inhibition of LDL oxidation and subsequent cytotoxicity. It is possible that either hydrogen peroxide or superoxide anion is responsible for the oxidation of LDL. Alternatively, hydrogen peroxide and superoxide anion may both be involved in the oxidation of LDL. Hydroxyl radical may be generated in the presence of hydrogen peroxide and superoxide anion according to the Haber-Weiss reaction (29). Our results have shown that mannitol, a known hydroxyl radical scavenger (17), was ineffective in protecting LDL from oxidation. It remains unclear, however, whether other hydroxyl radical scavengers would effectively protect LDL against oxidation.

The identity of the cytotoxic oxidized lipid carried by LDL and the mechanism of its action are as yet undetermined. While the assay for TBARS indicates oxidation, there is no conclusive evidence that the toxic substance(s) and the substance(s) responsible for a positive TBARS reading are the same. For example, oxidized sterols that have been found to be toxic to aortic smooth muscle cells in culture (30) may be generated by LDL oxidation and carried by LDL to the cells. The results showing that the TBARS assay at pH 1 provided a better indication of LDL cytotoxicity than the pH 3.5 assay indirectly implicate oxidation of polyunsaturated fatty acids in toxic LDL formation because the assay performed at the lower pH has greater sensitivity to hydroperoxides of polyunsaturated fatty acids than does the assay at pH 3.5 (24).

In our experimental system, LDL that is toxic to cells is formed by allowing oxidation to occur by dialysing the LDL without antioxidant protection. It is known, however, that the existence of lipid peroxides on lipoproteins occurs in certain in vivo conditions as well. Increased levels of lipid peroxides in the plasma of diabetic patients (31, 32) and in the plasma of rats with experimentally induced diabetes (33) have recently been reported. Serum from diabetic rats is toxic to cultured bovine endothelial cells with the toxicity localized to the very low density lipoprotein fraction (34). In addition, lipid peroxides are reported to be elevated in rats with experimentally induced myocardial necrosis (35) and in the arterial walls of humans with an increasing degree of atherosclerosis (36). If the lipid peroxide-associated cytotoxicity we have observed in vitro has an in vivo analog, lipoprotein-borne lipid peroxides may be an important link between oxidative free radical generation and tissue damage accompanying certain diseases.

The authors gratefully acknowledge the funding provided for these studies by the Whitaker Foundation (Camphill, PA). G.M.C. is the recipient of an NIH Research Career Development Award (HL 00359); J.R.H., a fellowship from the American Heart Association, Northeast Ohio Affiliate; D.W.M., a predoctoral training grant fellowship (GM-07535). Human foreskins from circumcisions were obtained through the Perinatal Clinical Research Center (NIH USPHS M01 RR00210), Cleveland Metropolitan General Hospital. The cooperation of Dr. Herbert K. Naito, head of the Lipid and Lipoprotein Laboratories, Cleveland Clinic Foundation, is gratefully ac-

knowledged. The authors wish to thank Ms. Kathy G. Holley and Ms. Margot K. Reed for expert technical assistance.

Manuscript received 13 January 1983 and in revised form 29 March 1983.

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