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THE SUSCEPTIBILITY OF LOW DENSITY LIPOPROTEIN TO CHEMICAL OXIDATION IS CLOSELY RELATED TO PRONENESS TO BIOLOGICAL MODIFICATION

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U937 is a monocytic cell line dependent on low density lipoprotein (LDL) receptor-mediated uptake of cholesterol for proliferation. However, exposure of U937 cells to LDL also results in an oxidative modification of LDL. We report here that the oxidative modification of LDL by U937 cells results in inhibition of growth and cell death. This finding suggests that analysis of U937 cell growth in presence of LDL may be used to determine the susceptibility of LDL to biological oxidative modification. There was an inverse association between the effect of LDL on U937 cell growth and the rate of degradation of U937 cell-modified LDL in mouse peritoneal macrophages ($r=-0.82$, $p<0.05$) suggesting a coupling between proneness of LDL to develop cytotoxicity and affinity for scavenger receptors. In a group of young post-infarction patients ($n=18$) the susceptibility of LDL to chemical oxidation as determined by analysis of the lag phase for formation of conjugated diens in presence of copper ions was compared with the biological modification of LDL as assessed by analysis of U937 cell growth in presence of LDL. The results demonstrated a close relation between the estimates of chemical oxidation and biological modification ($r=0.86$, $p<0.005$) suggesting that LDL, which is prone to become oxidised by copper also is more prone to become modified by cells in vivo.

KEYWORDS: oxidation, low density lipoprotein, atherosclerosis.

INTRODUCTION

Elevation of low density lipoprotein (LDL) cholesterol is a well established risk factor for development of coronary artery disease[1] but the mechanisms by which LDL promotes atherogenesis are not fully understood. High concentrations of LDL have a cytotoxic effect on cultured endothelial cells[2,3] suggesting that LDL may promote the formation of atherosclerotic lesions by inducing damage to arterial tissue. Experiments using antioxidants have shown that the cytotoxicity of LDL is due to lipid oxidation products formed during exposure of LDL to cultured cell.[4] The oxidative modification of LDL by cells has been found to involve either a release of superoxide ions[5] or a lipooxygenase-dependent process.[6] The oxidation results in a degradation of apolipoprotein B, uptake of LDL in macrophages by the scavenger receptor pathway and formation of foam cells.[7] With the use of antibodies directed against LDL oxidised in vitro, Palinsky and coworkers[8] were able to demonstrate the presence of oxidised

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LDL in atherosclerotic lesions which suggests that oxidation of LDL occurs also in vivo. Based on these findings it has been proposed that oxidative modification of LDL may be an important process in the early stages of atherosclerosis. This hypothesis has been further supported by animal studies demonstrating that probucol, a lipid-lowering drug with antioxidant properties, has a more pronounced antiatherogenic effect than other equally potent lipid-lowering drugs.[9,10]

Against this background considerable interest has focused on methods to quantify the susceptibility of LDL to oxidation in clinical studies. Determination of the lag phase for initiation of formation of conjugated diens in isolated LDL samples exposed to copper ions has now become the most widely used assay.[11] However, it may be argued that the use of copper ions is unphysiological and that this assay poorly reflects the LDL oxidation induced by cells in the arterial wall in vivo. In the present study we compared the susceptibility to oxidative modification of LDL in presence of copper ions as determined by the lag phase for dien formation with the proneness to biological modification of LDL by U937 cells in culture. U937 is a monocytic cell line derived from a histiocytic lymphoma.[12] The cells lack the ability to synthesize cholesterol endogenously and as a consequence they require addition of exogenous cholesterol in order to proliferate.[13] The major pathway for cholesterol uptake in U937 cells is by the LDL receptor.[14] However, U937 cells have also been found to have the capacity to oxidatively modify LDL.[15] We demonstrate here that the oxidative modification of LDL by U937 cells decreases U937 cell growth and viability. Analysis of U937 cell growth in the presence of LDL may thus be used to estimate the proneness of LDL to biological modification.

METHODS

Patients

LDL was isolated from 18 patients who had suffered a first myocardial infarction before the age of 45. Metabolic studies were performed 4–6 months after the acute event when acute phase reactions due to myocardial damage had subsided. At the time of the investigation none of the patients were on lipid-lowering drugs, but all had been informed about a lipid-lowering diet in connection with the first visit to the outpatient clinic 6 weeks after admission to the coronary care unit. Basic characteristics of the patients are shown in Table 1.

Lipoprotein determinations and isolation of LDL

Blood samples for lipoprotein analyses and isolation of LDL were taken between 8.00 and 9.00 a.m. after 12 hr of fasting, during which time smokers were asked to refrain from smoking. Venous blood was drawn into pre-cooled vacutainer tubes containing Na_2EDTA (1.4 mg/ml) and placed in icebath. Plasma was then recovered by low speed centrifugation (1400 × g, 20 min, +1°C) and kept at this temperature throughout the preparation procedures. The major plasma lipoproteins (very low density lipoprotein (VLDL), LDL and high density lipoprotein (HDL)) were determined by a combination of preparative ultracentrifugation and precipitation of apolipoprotein B-containing lipoproteins followed by lipid analyses as described[16]. LDL for compositional analyses and determination of resistance to oxidative modification was prepared by cumulative rate ultracentrifugation in a density gradient.[17] Free and total cholesterol (14106–14108

TABLE 1
Basic characteristics of the patient group.

	No. of subject in group or mean ±SD
Age (yrs)	40.5 ±3.7
Sex	
Males	17
Females	1
B-blocker medication	17
Hypertension*	5
Smoking habits†	
Non smokers	5
Former smokers	12
Present smokers	1
Lipoprotein phenotype	
Normal	6
IIa	4
IIb	1
IV	7

*A medical history of hypertension before the myocardial infarction.
†Non smokers = Never smoked or stopped at least two years before the myocardial infarction. Former smokers = Stopped smoking at the time of the myocardial infarction.

Merck Diagnostica, Darmstadt, Germany) and triglycerides (877557 Boehringer Mannheim Diagnostica, Germany) were determined with the use of enzymatic methods.

Oxidation of LDL by copper

Isolated LDL was dialysed in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl for 15 hr at 4°C to remove EDTA. Copper-mediated oxidation of LDL was performed by incubating 0.2 mg/ml of EDTA-free LDL in medium F-10 containing 5×10^{-6}M $CuSO_4$ overnight at 37°C. Each preparation of oxidised LDL was used for cell experiments within one week of the oxidation. LDL concentrations are given as protein LDL/ml.

Determination of proneness of LDL to biological modification by U937 cells

U937 cells[12] were grown in medium RPMI 1640 (GIBCO BRL, Glasgow, Scotland) supplemented with 10% heat inactivated fetal calf serum (GIBCO BRL) and 50 μg/ml of gentamycin sulphate and kept in an atmosphere of 5% CO_2 in air. Fresh medium was added twice weekly. The cells were then transferred to serum-free RPMI 1640 medium and seeded out in 12-multi well plates at a concentration of 3×10^5 cells/ml. Lipoprotein and superoxide dismutase (Boehringer Mannheim, Mannheim, Germany) were added at indicated concentrations and cell growth analysed by determining the final cell number after 72 hr using an electronic cell counter (VDA 140, Analys Instrument AB, Stockholm, Sweden). Cell viability was determined by analysing the ability to exclude the dye trypan blue. Formation of lipid peroxides in lipoproteins exposed to U937 cells was measured in terms of thiobarbituric acid reactive material and expressed as malondialdehyde equivalents, as described by Yagi.[18] Iodination of

LDL and determination of the rate of degradation of ^{125}I-labelled LDL in mouse peritoneal macrophages was performed as described.[19] The cell-specific effects on modification of LDL were calculated by subtracting the increases in macrophage degradation and TBARS occurring in cell-free dishes.

Analysis of susceptibility to LDL oxidation in presence of copper ions

Determination of the susceptibility of LDL to oxidation in vitro was performed essentially as described by Esterbauer et al.[11] LDL was dialysed in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl (dialysis buffer) for 15 hr at 4°C to remove EDTA and then diluted in dialysis buffer to a final concentration of 25 μg/ml. Oxidation was initiated by addition of a freshly prepared aqueous $CuSO_4$ solution to a final concentration of 1.66 μM. The kinetics of the LDL oxidation was followed by monitoring of the change in absorbance at 234 nm and 20°C in a Beckman DU-64 spectrophotometer. The initial absorbance at 234 nm was set to zero and the change in absorbance from baseline level was recorded every 5 min during a period of 4 hr. The lag phase for formation of conjugated diens was defined as the intercept of the tangent of the slope of the absorbance curve in propagation phase with the base line and expressed in min.

Statistics

Conventional methods were used for calculation of means and standard deviations. Coefficients of skewness and kurtosis were calculated to test deviations from a normal

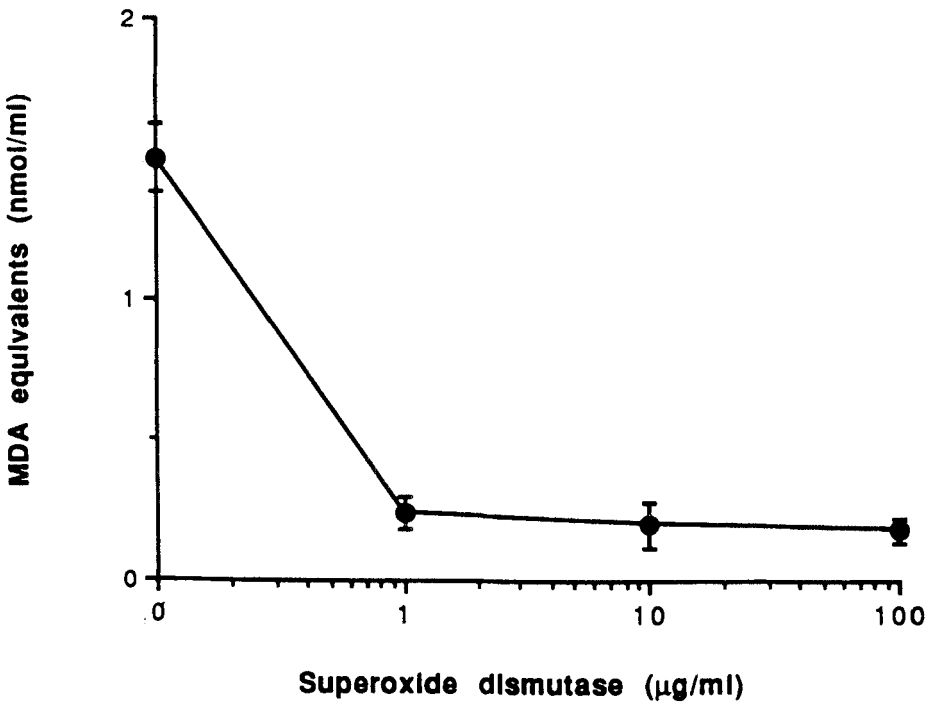

FIGURE 1. Effect of superoxide dismutase on U937 cell oxidation of LDL. U937 cells (3×10^5 cells/ml) were incubated with 25 μg/ml of LDL in serum-free RPMI medium for 24 hr. The amount of TBARS present in the medium was then determined and expressed as malondialdehyde equivalents. Each value represents the mean±SD of triplicate determinations.

distribution. Relations between lag phase and lipoprotein variables on the one hand and U937 cell survival on the other hand were analysed by compution of Spearman rank correlation coefficients.

RESULTS

Effect of LDL oxidation on viability and growth of U937 cells

Incubation of 25 μg/ml LDL with a U937 cell clone resulted in oxidative modification of LDL as evidenced by an increase in LDL TBARS content from 0.12 to 1.50 nmol malondialdehyde equivalents/ml. The oxidative modification of LDL by this U937 cell clone was inhibited by addition of 1 μg/ml of superoxide dismutase (Figure 1). To exclude the possibility that the superoxide dismutase exerted its effects through unspecific mechanisms, superoxide dismutase was compared to inactivated superoxide dismutase. The increase in TBARS determined after incubation of U937 cells with 25 μg/ml LDL in the presence of 10 μg/ml of superoxide dismutase was 24 % ± 2.5 of control, but 102% ± 3.7 when 10 μg/ml of inactivated superoxide dismutase was added.

To study how oxidised LDL affects U937 growth, cells were cultured in serum-free RPMI 1640 medium in presence of increasing concentrations of copper oxidised LDL for 72 hr. In contrast to native LDL, oxidised LDL was found to lack the ability to stimulate growth of U937 cells, and at concentrations above 10 μg/ml cytotoxic effects were observed (Figure 2). Addition of superoxide dismutase effectively inhibited the

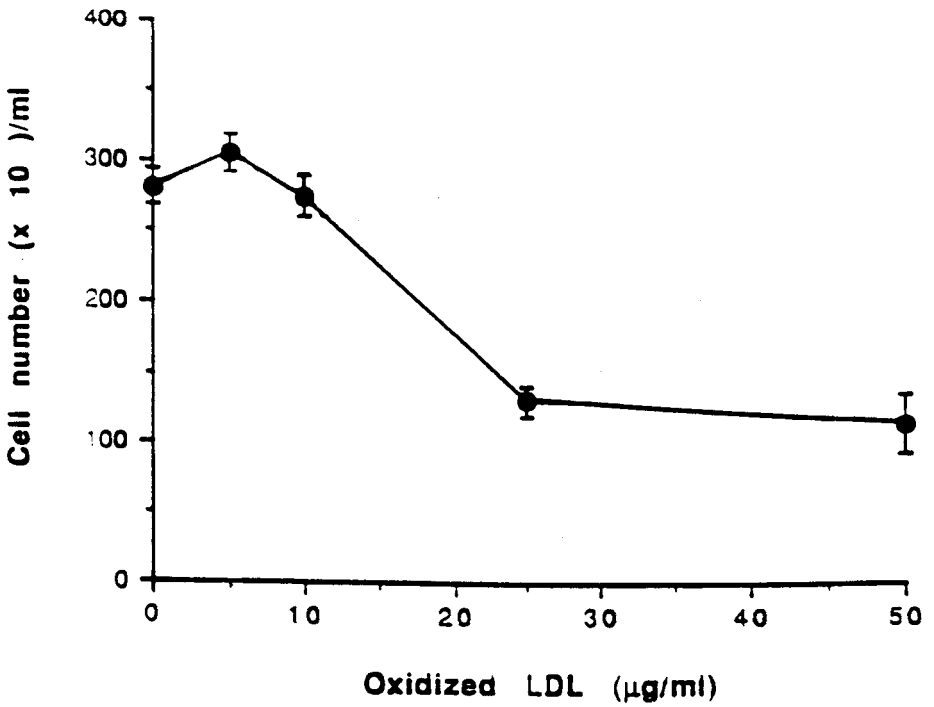

FIGURE 2. Effect of copper ion oxidised LDL on U937 cell growth. U937 cells were seeded in multiwell plates at a concentration of 3×10^5 cells/ml and grown in serum-free medium containing different concentrations of copper ion oxidised LDL for 72 hr. The final cell number was determined using an electronic cell counter. Each value represents the mean±SD of triplicate determinations.

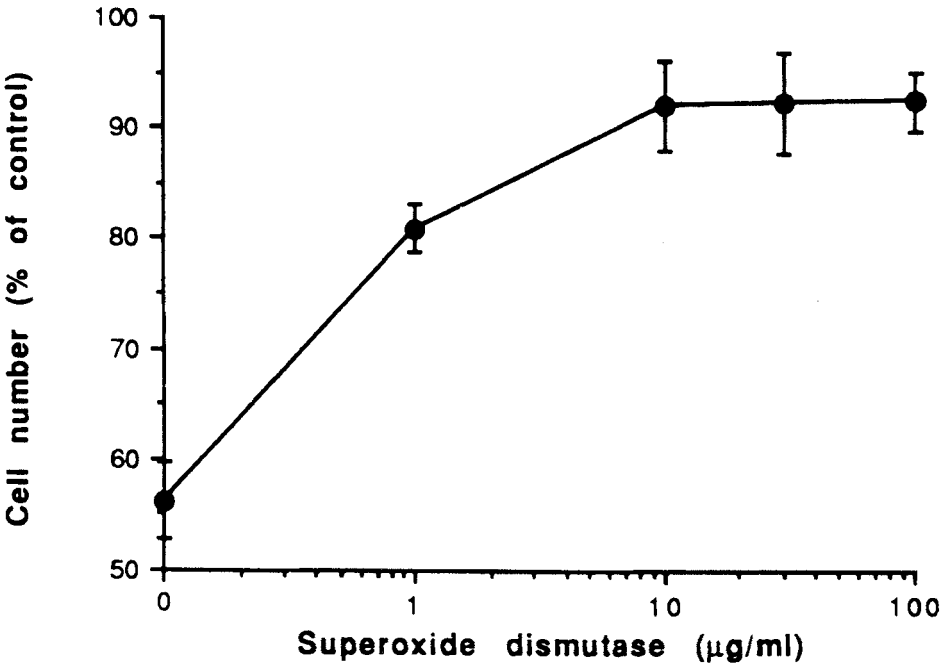

FIGURE 3. Effect of superoxide dismutase on U937 cell growth in presence of oxidised LDL. U937 cells were seeded in multiwell plates at a concentration of 3×10^5 cells/ml and grown in serum-free medium containing 25 μg/ml of copper ion oxidised LDL and different concentrations of superoxide dismutase for 72 hr. The final cell number was then determined and expressed as percent of growth of cells cultured without oxidised LDL. Values are given as mean±SD of triplicate determinations.

cytotoxic effect of copper-oxidised LDL on U937 cells (Figure 3). In accordance with earlier observations, addition of low concentrations of LDL stimulated the growth of U937 cells kept in serum-free medium. Exposure of U937 cells to 5 μg/ml of LDL resulted in a 40% increase in cell number over a three-day period (Figure 4). However, at LDL concentrations exceeding 10 μg/ml the growth-stimulatory effect of LDL disappeared, and at concentrations of 25 μg/ml and above signs of cell death could be observed. Addition of superoxide dismutase at a concentration of 10 μg/ml to the LDL containing medium markedly enhanced the growth stimulatory effect of LDL and blocked the cytotoxic effect of high LDL concentrations (Figure 4). After exposure of U937 cells to 5 μg/ml of LDL for 72 hr, around 80% of the cells were viable as assessed by their ability to exclude trypan blue, whereas only 40% of the cells exposed to 50 μg/ml of LDL were viable by this criterion (Figure 5).

U937 cell modification of LDL in relation to effect on cell growth and degradation in cultured macrophages

The finding that U937 cells may oxidatively modify LDL and that the cell-modified LDL in turn inhibits further growth of the cells suggested that determination of U937 cell growth in the presence of LDL may be used to analyse the susceptibility of LDL to biological modification. To further study this possibility LDL was isolated from six young post-infarction patients and labelled with ^{125}I. U937 cells (3×10^5 cells/ml) were

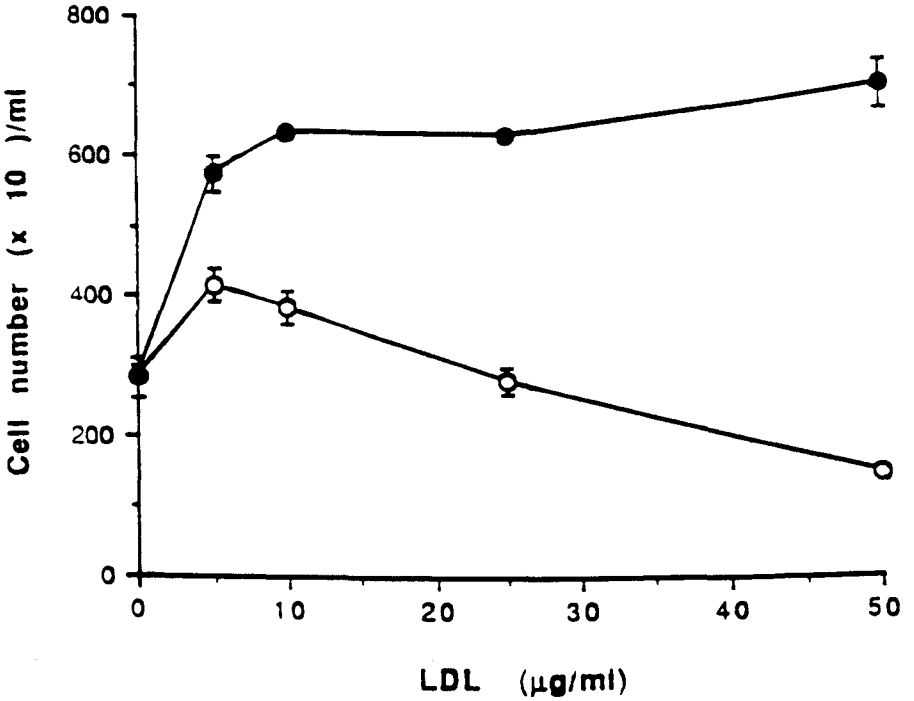

FIGURE 4. Effect of superoxide dismutase on U937 cell growth in presence of native LDL. U937 cells were seeded in multiwell plates at a concentration of 3×10^5 cells/ml and grown in serum-free medium containing different concentrations of native LDL for 72 hr with (●) or without (○) the addition of 10 µg/ml of superoxide dismutase. The final cell number was then determined and expressed as mean±SD of triplicate determinations.

then incubated with 20 µg/ml of ^{125}I-labelled LDL for three days. The final cell numbers were found to vary between 68.7 and 111.3% (mean±SD; 92.5±22.2%) of the initial cell number. The culture medium was then removed and the concentration of I^{125}-labelled LDL adjusted to 10 µg/ml. Degradation of I^{125}-labelled, cell-modified LDL was determined in cultured mouse peritoneal macrophages. The rate of degradation of the LDL samples varied between 205 and 2195 ng LDL degraded/mg cell protein/4 hr. There was an inverse relation between the effect of LDL on U937 cell growth and the rate of degradation in macrophages ($r=-0.82$, $p<0.05$). The amount of TBARS formed in LDL during the 72 hr exposure to U937 cells showed a close inverse relation to the effect of LDL on U937 cell survival ($r=-0.93$, $p<0.01$).

Relation between U937 cell modification of LDL and oxidation of LDL induced by cupper ions

In samples isolated from 18 post-infarction patients the susceptibility of LDL to chemical oxidation in vitro was determined by measuring the lag phase for formation of conjugated diens in presence 1.66 µM $CuSO_4$ and the proneness to biological modification of LDL by determining the growth of U937 cells incubated with 25 µg/ml of LDL for three days. There was a close association between the lag phase for formation of conjugated diens in the LDL samples exposed to copper ions and U937

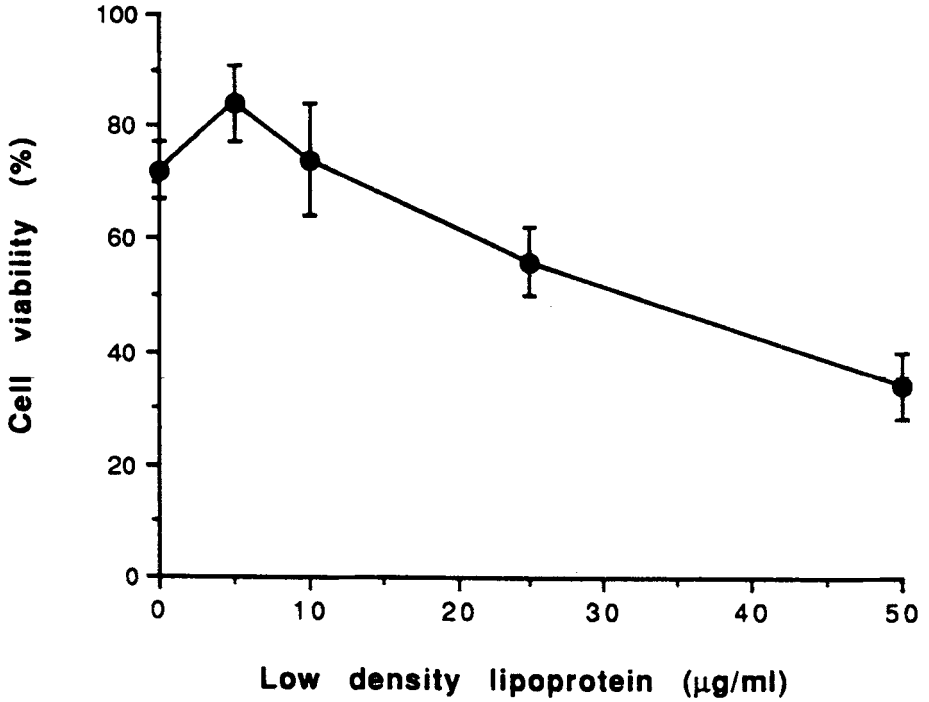

FIGURE 5. Effect of LDL on U937 cell viability. U937 cells were seeded in multiwell plates at a concentration of 3×10^5 cells/ml and grown in serum-free medium containing different concentrations of LDL for 72 hr. Cell viability was then analysed by determining the fraction of cells capable of excluding trypan blue.

cell growth in presence of LDL ($r=0.86$, $p<0.005$; Figure 6, Table 2). The lag phase for dien formation showed an inverse association with LDL triglyceride content ($r=-0.54$, $p<0.05$). U937 cell growth also tended to be inversely associated with the LDL triglyceride level. The effect of LDL on U937 cell survival was not related to the LDL cholesterol content (Table 2). To study the time-dependent variation in oxidation susceptibility, LDL was isolated from six healthy controls at an interval of one week. The correlation coefficient between the lag phase estimates of the first and second LDL samples was 0.92, implicating that the susceptibility of LDL to oxidative modification was fairly constant.

DISCUSSION

The present findings confirm earlier studies in demonstrating that U937 cells have the capacity to oxidatively modify LDL[15] and show that the subsequent exposure of the cells to oxidised LDL decreases cell growth and viability. To a large extent the decreased growth rate is due to a cytotoxic effect of oxidised LDL on the cells. However, as native LDL is a potent stimulus to serum-free growth of U 937 cells[14] it cannot be excluded that part of the growth-inhibitory effect of LDL modified by oxidation is due to a decreased number of LDL particles available for LDL receptor-

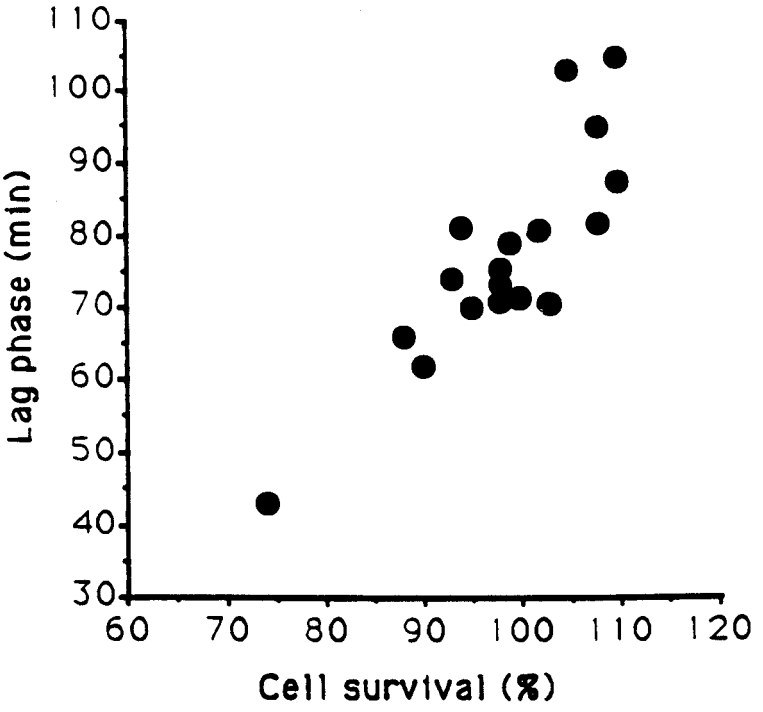

FIGURE 6. Correlation between susceptibility of LDL to chemical oxidation as assessed by the lag phase for dien formation in presence of copper and proneness to biological modification as determined by U937 cell growth in presence of LDL. U937 cell growth is expressed as percent of the growth induced by LDL from a normolipidemic control.

mediated uptake. This possibility may be of relevance for the application of the U937 method to study the receptor binding properties of LDL.[14] In view of the present results, a decreased ability of LDL to promote proliferation of U937 cells may not only be due to genetic defects in apolipoprotein B but also to degradation of apolipoprotein B as a result of lipid oxidation. In order to avoid this confounding, factor the U937 LDL binding assay should be run in the presence of antioxidants. The finding that superoxide dismutase markedly enhances the growth stimulatory effect of LDL indicates that U937 cells oxidise LDL by a superoxide-dependent mechanism and that LDL which

TABLE 2
Spearman Rank correlation coefficients between U937 cell growth, lag phase for dien formation and plasma lipoprotein lipid concentrations.

	Lag time	VLDL		LDL		HDL	
		Chol	TG	Chol	TG	Chol	TG
U937 cell growth	–0.792**	–0.102	–0.100	–0.170	–0.436	0.045	–0.219
Lag phase		–0.105	–0.069	–0.294	–0.543*	–0.144	–0.314

Chol; cholesterol, TG; triglycerides, VLDL; very low density lipoprotein, LDL; low density lipoprotein, HDL; high density lipoprotein
*P<0.05; **P<0.005.

has become oxidised by the cells becomes cytotoxic and inhibits further growth of the cells. It is possible that SOD inhibits the toxic effects of oxidised LDL by removal of the superoxide radical, which is present in oxidised LDL. Our findings are in accordance with recent investigations reporting that cell-induced oxidation of LDL is related to superoxide radical production[20] However, other reports indicate that cells may oxidize LDL by other mechanisms than superoxide radical.[21] LDL modified by U937 cells has several characteristics in common with LDL oxidised by exposure to copper ions. It contains increased amounts of TBARS and is degraded at an increased rate by macrophages. The LDL samples which after exposure to U937 cells showed the highest TBARS content and the most rapid rate of macrophage degradation also were the most cytotoxic. These findings indicate that analysis of U 937 cell growth in the presence of LDL may be used as an estimate of the susceptibility of LDL to biological modification.

It has become increasingly evident that quantitative analysis of plasma lipoprotein lipid concentrations provides incomplete information on the atherogenicity of plasma lipoproteins. Development of assays for analysis of other aspects of lipoproteins may help to further identify individuals at increased risk of developing coronary heart disease (CHD). Since there is accumulating experimental evidence that oxidation of LDL may play an important role in atherogenesis,[22] several clinical studies have been designed to evaluate the role of lipid oxidation in the development of human atherosclerosis. However, the results of these studies have been equivocal. Several investigators have been unable to identify any relations between plasma antioxidant concentrations and CHD mortality.[23,24] In contrast, Riemersma and coworkers have demonstrated decreased plasma levels of vitamin E in patients with angina pectoris.[25] An approach presently used by many investigators is to analyse the susceptibility of LDL to oxidation by determining the lag phase for formation of conjugated diens in LDL exposed to copper ions in vitro. Studies from our group indicate that a significant association exists between the ability of LDL to resist oxidation as determined by this method and the severity of angiographically assessed coronary atherosclerosis in young post-infarction patients.[26] However, it has been argued that copper-induced oxidation of LDL represents an unphysiological process with little relevance for the oxidation that may occur in the artery wall in vivo. In this respect the present finding demonstrating a close association between the susceptibility of LDL to copper oxidation and the biological modification of LDL by U937 cells suggests that the analysis of lag phase for dien formation in presence of copper ions also reflects the proneness of LDL to become modified by cells in vivo. Furthermore, LDL which is prone to oxidation also is more prone to become cytotoxic which might represent a possible mechanism linking susceptibility to oxidation to coronary atherosclerosis.

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