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S Parthasarathy, … , R C Pittman, D Steinberg

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Research Article

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Probucol Inhibits Oxidative Modification of Low Density Lipoprotein

Sampath Parthasarathy, Stephen G. Young, Joseph L. Witztum, Ray C. Pittman, and Daniel Steinberg
Department of Medicine, University of California, San Diego, La Jolla, California 92093

Abstract

Previous studies have established that low density lipoprotein (LDL) incubated with endothelial cells (EC) undergoes extensive oxidative modification in structure and that the modified LDL is specifically recognized by the acetyl LDL receptor of the macrophage. Thus, in principle, EC-modified LDL could contribute to foam cell formation during atherogenesis. Oxidatively modified LDL is also potentially toxic to EC. The present studies show that addition of probucol during the incubation of LDL with EC prevents the increase in the electrophoretic mobility, the increase in peroxides, and the increase in subsequent susceptibility to macrophage degradation. It has also been shown that oxidation of LDL catalyzed by cupric ion induces many of the same changes occurring during EC modification. Addition of probucol (5 μM) also prevented this copper-catalyzed modification of LDL. Most importantly, samples of LDL isolated from plasma of hypercholesterolemic patients under treatment with conventional dosages of probucol were shown to be highly resistant to oxidative modification either by incubation with endothelial cells or by cupric ion in the absence of cells. The findings suggest the hypothetical but intriguing possibility that probucol, in addition to its recognized effects on plasma LDL levels, may inhibit atherogenesis by limiting oxidative LDL modification and thus foam cell formation and/or EC injury. Other compounds with antioxidant properties might behave similarly.

Introduction

Accumulation of lipid-loaded "foam cells" in the subendothelial space is one of the early events in atherogenesis. Evidence has recently accumulated suggesting that many of these cells represent monocyte/macrophages that have entered by penetration of the endothelial lining (1–3). These cells can take up and degrade native low density lipoprotein (LDL) but at a rather low rate and generally without marked accumulation of cholesterol (4). In contrast, they take up and degrade certain chemically modified forms of LDL at much higher rates (4–6). While these chemically modified forms have yet to be demonstrated in vivo, a biologically modified form of LDL that reacts with the acetyl LDL receptor can be generated by incubation of native LDL with cultured endothelial cells (EC)[1] or arterial smooth muscle cells (7, 8). This modification is accompanied by a number of striking changes, including a marked increase in electrophoretic mobility, an increase in density, hydrolysis of lecithin to lysolecithin, degradation of the apoprotein B, and the generation of peroxides (7–10). Addition of alpha tocopherol or butylated hydroxytoluene, effective free-radical scavengers, inhibited peroxidation and most of the other changes as well (9). Most of the same changes can be induced by oxidizing LDL in the presence of cupric ions in the absence of cells (9–13). Whether the modification is copper catalyzed or induced by incubation with cells, the oxidized LDL is toxic to some cultured cells, including EC (13–15), and inhibits the motility of the macrophage (16). Thus, there are a number of ways in which EC modification of LDL (or oxidation by other mechanisms) might contribute to atherogenesis: (*a*) by favoring lipid accumulation in foam cells; (*b*) by damaging the endothelial lining; and (*c*) by favoring the accumulation of macrophages in the subendothelial space due to its inhibitory effect on macrophage motility.

The present studies show that probucol, a drug widely used in treatment of hypercholesterolemia, when added to incubations of LDL with cultured EC, blocks all of the cell-induced changes, including the conversion to a form recognized by the acetyl LDL receptor. In these in vitro studies, probucol was effective at concentrations even below the plasma concentrations reached during conventional clinical use of the drug. The drug also blocked the oxidative changes of LDL induced by cupric ion in the absence of cells. Finally, LDL isolated from the plasma of patients under treatment with usual doses of probucol (500 mg b.i.d.) resisted both EC modification and copper ion-catalyzed oxidation.

Methods

Rabbit aortic EC (a cell line developed by Dr. V. Buonassissi, Dept. of Biology, U. C. San Diego) were grown in Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) supplemented with 15% fetal bovine serum as described (7). Resident peritoneal macrophages were harvested from female Swiss-Webster mice by lavage and used for measurements of degradation of LDL as described (9). LDL (d = 1.019–1.063 g/ml) was isolated from fresh human plasma and labeled with carrier-free ^{125}I (Amersham Corp., Arlington Heights, IL) either by a modification of the MacFarlane method (17) or using 1,3,4,6-tetrachloro-3,6-di-phenylglycouril, a water-insoluble oxidizing agent (Iodogen, Pierce Chemical Co., Rockford, IL) (18). Probucol [4,4′-(isopropylidenedithio)*bis*(2,6-di-*t*-butylphenol)] was provided by Merrell Dow Pharmaceuticals, Indianapolis, IN.

The extent of lipid peroxidation was measured in terms of thiobarbituric acid-reactive (TBA) products expressed as malondialdehyde equivalents (10). ^{125}I-LDL (100 μg/ml) was modified by incubations with cultured EC in F-10 medium for 24 h at 37°C. At the end of the modifying

Address correspondence to Dr. Steinberg.

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1. *Abbreviations used in this paper:* EC, endothelial cell(s); TBA, thiobarbituric acid-reactive.

incubation, enough medium was transferred to a macrophage culture to bring the final concentration of ^{125}I-LDL to 10 μg/ml. After a 5-h incubation with the macrophages at 37°C in Dulbecco's modified Eagle's medium, the trichloroacetic acid-soluble ^{125}I was measured and corrected for trichloroacetic acid-soluble ^{125}I in an aliquot incubated 5 h without macrophages.

LDL (d = 1.025–1.063 g/ml) was isolated by ultracentrifugation from individual plasma samples from patients with familial hypercholesterolemia under treatment with probucol (500 mg twice daily) in connection with a clinical trial of the efficacy of the drug. Each patient had given fully informed consent. Control samples were taken from untreated hypercholesterolemic patients or from normolipidemic volunteers. Each LDL sample was individually iodinated and evaluated for its susceptibility to EC modification and to copper-catalyzed oxidation as described. Levels of probucol in whole plasma and in LDL samples were determined in the laboratories of Merrell Dow Pharmaceuticals using high performance liquid chromotography.

Results

As shown in Table I, LDL incubated with EC for 24 h showed a 10-fold increase in peroxides (TBA-reactive materials) and a fivefold increase in rate of degradation by macrophages, in agreement with previous studies (7–10). The addition of probucol, even at concentrations as low as 1 μM, strongly inhibited these modifications, and at 5 μM inhibited them completely. To show that probucol did not interfere with the assay of peroxides nor the subsequent degradation by macrophages, the probucol in some cases was added at the end of the incubation with EC, just before the transfer of the LDL to macrophage cultures. This had no effect.

As shown in Table II, probucol also inhibited the peroxidation and the biological modification of LDL incubated in the absence of cells but in the presence of 5 μM Cu^{++}. The inhibition of oxidation was considerable—>50%—but not as striking as the inhibition of EC-induced oxidation (Table I); the inhibition of the increase in macrophage degradation was almost complete.

As shown in Table III, LDL isolated from the plasma of probucol-treated patients was also resistant both to EC modification and to Cu^{++}-induced modification. The former was inhibited completely; the latter was inhibited only partially, as in the case of probucol addition to LDL in vitro. Plasma probucol levels in the samples studied averaged 98 μM (range 60–114 μM; 31.2–59.3 μg/ml). The probucol levels were also determined in the LDL samples prepared by ultracentrifugation of these plasmas. The values ranged from 12.7 to 21.3 μg probucol/mg LDL protein, with an average value of 16.4. Since 100 μg LDL protein/ml were added in the EC modification incubation (see Table III), the final probucol concentration in that incubation averaged 1.64 μg/ml or 3.2 μM. Note, however, that the notion of "concentration" is not strictly applicable here, since probucol is virtually insoluable in aqeous media and will be present concentrated in the lipoprotein fraction. Similarly, in the studies described in Tables I and II, the probucol added to LDL-containing media probably partitioned primarily into the LDL.

Discussion

The possibility that free radicals and peroxidation may play a role in the pathogenesis of atherosclerosis has been proposed (19, 20), although the available evidence is indirect and limited. Certainly lipid peroxidation occurs in vivo and peroxidized lipids are found in atherosclerotic lesions (21). It has been shown that intravenous injection of peroxidized free fatty acids can damage EC (20), but the relevance of this remains to be established. There is no completely satisfactory evidence that would link peroxidation causally to lesion initiation or progression. The finding that cell-modified (oxidized) LDL is recognized by the macrophage acetyl LDL receptor (8, 9), suggests such a linkage—by favoring development of foam cells. Cell-modified LDL could also contribute by damaging EC, as it has been shown to do in vitro (13–15). Finally, the recent demonstration that cell-modified LDL inhibits macrophage motility suggests another mechanism, namely, that it could favor accumulation of foam cells by inhibiting their exit from the subendothelial space (16).

The studies reported here show that probucol is a highly effective inhibitor of the oxidative, cell-mediated modification of LDL. Added in vitro it was effective at levels well below those reached in the plasma during conventional treatment of hyper-

Table I. Probucol Added In Vitro Inhibits EC Modification of LDL

LDL sample	TBA-reactive materials	Macrophage degradation (μg/5 h/mg)
	nmol/ml	
Native, unincubated	0.34	1.77
Incubated 24 h without cells	0.98	0.64
Incubated 24 h with cells	4.46	9.62
+1.0 μM probucol	2.71	4.70
+2.5 μm probucol	1.09	0.79
+5.0 μm probucol	1.10	0.73
+5.0 μm probucol added at the end of incubation	4.56	10.40

200 μg ^{125}I-LDL was incubated for 24 h with rabbit aortic EC in 2 ml of F-10 medium at 37°C. Probucol was added in 20 μl of ethanol to achieve the indicated final concentrations; ethanol alone was added to controls. Total TBA-reactive material was determined on one aliquot of medium. Another aliquot was transferred to a dish containing mouse peritoneal macrophages to yield a final ^{125}I-LDL concentration of 10 μg/ml and to a no-cell control dish. After 5 h incubation at 37°C, trichloroacetic acid-soluble ^{125}I, corrected for no-cell control values, was determined as a measure of LDL degradation. Results represent averages from two separate experiments in each of which duplicate dishes were incubated for each specified condition and separately assayed.

Table II. Probucol Inhibits Oxidation and Biological Modification of LDL Catalyzed by Cu^{++} in the Absence of Cells

LDL sample	TBA-reactive materials	Macrophage degradation (μg/5 h/mg)
	nmol/ml	
Unincubated	0.71	0.45
Incubated 24 h (no additions)	1.82	0.32
+5.0 μM Cu^{++}	8.12	8.37
+5.0 μm Cu^{++} and 5.0 μm probucol	3.47	1.10

200 μg ^{125}I-LDL was incubated at 37°C for 24 h in 2 ml of F-10 medium with additions as indicated. Conditions and analysis as in the legend to Table I. Results again represent averages for two separate experiments.

Table III. LDL from the Plasma of Probucol-treated Patients Resists Cell-induced and Cu^{++}-induced Modification

LDL samaple	TBA-reactive materials		Macrophage degradation	
	LDL from untreated controls	LDL from probucol-treated patients	LDL from untreated controls (μg/5 h/mg)	LDL from probucol-treated patients (μg/5 h/mg)
	nmol/ml	*nmol/ml*		
Unincubated	0.54±0.20	0.55±0.08	1.31±0.27	1.27±0.05
Incubated 24 h without cells	2.27±0.58	1.40±0.19	1.30±0.22	1.1±0.24
Incubated 24 h with Cu^{++}	5.29±0.44	2.99±0.51	11.85±1.30	3.56±2.30
Incubated 24 h with cells	3.90±0.54	1.01±0.16	9.34±2.40	1.14±0.28

LDL samples were prepared from the plasma of four untreated controls and from that of four patients under treatment with probucol (500 mg twice daily). Incubations and analyses as described under Table I were carried out in duplicate for each sample and the results averaged. Values represent group means±SE.

cholesterolemia. Most important, the LDL isolated from the plasma of patients treated with conventional dosages of probucol was highly resistant to oxidative modification. Probucol, as might be anticipated from its highly lipophilic properties, is transported in plasma primarily in lipoproteins, mostly in LDL and VLDL (22). Thus, it would be strategically positioned to offer maximum protection of the lipoproteins against in vivo peroxidative processes. In previous studies from this laboratory (18), it was shown that LDL catabolism was accelerated in probucol-treated rabbits, in agreement with reports of increased fractional catabolic rate of LDL in patients treated with the drug (23, 24). However, the increased catabolism was only seen when the LDL was prepared from probucol-treated rabbits, and then it was seen whether the recipient was under treatment with probucol or not. In short, the studies indicated that the LDL itself was modified somehow as a result of probucol treatment. Just what change is critical has yet to be determined, but it could be related, again, to the fact that probucol and its metabolites are transported in LDL and may modify the metabolism of the molecule.

If the cell-induced modification of LDL is of relevance in atherogenesis, then we have the rather unusual situation that a drug introduced and used because of its effectiveness in lowering plasma cholesterol levels may have further antiatherogenic effects through quite different mechanisms. The question is whether it can be shown to be antiatherogenic over and above what would be expected on the basis of its cholesterol-lowering effect. Kritchevsky et al. (25) found that probucol at 1% in the diet of rabbits on a 2% cholesterol-6% corn oil diet significantly reduced the severity of aortic atherosclerosis, but to a degree compatible with the degree of plasma cholesterol lowering. However, atherosclerosis in this model is probably due primarily to an elevation of beta VLDL, which does not need to be modified; i.e., it is avidly taken up by the macrophage via the specialized beta VLDL receptor (26). The findings reported here provide us with a tool to use in testing more directly the hypothesis that oxidative modification of LDL is of consequence in the development of atheromata.

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