A novel mechanism by which probucol lowers low density lipoprotein levels demonstrated in the LDL receptor-deficient rabbit

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Abstract Treatment of low density lipoprotein (LDL) receptor-deficient rabbits (WHHL rabbits) with probucol (1% w/w in a chow diet) lowered their LDL-cholesterol levels by 36%, consonant with the reported effectiveness of the drug in patients deficient in the LDL receptor. Initial studies of LDL fractional catabolic rate (FCR) using ^{125}I-labeled LDL prepared from the serum of untreated WHHL rabbits showed no difference between probucol-treated WHHL rabbits and untreated WHHL rabbits. When, however, ^{125}I-labeled LDL was prepared from donor WHHL rabbits under treatment with probucol and injected back into them, the FCR was found to be increased by about 50% above that measured simultaneously using ^{131}I-labeled LDL prepared from untreated WHHL donors. The labeled LDL from probucol-treated donors was also metabolized more rapidly than that from untreated donors when injected into untreated WHHL rabbits or into untreated wild-type New Zealand White rabbits. Finally, it was shown that rabbit skin fibroblasts in culture degraded labeled LDL prepared from probucol-treated WHHL rabbits more rapidly than that prepared from untreated WHHL donors. This was true both for normal rabbit fibroblasts and also for WHHL skin fibroblasts, although the absolute degradation rates in the latter were, of course, much lower for both forms of LDL. The data indicate that a major mechanism by which probucol lowers LDL levels relates not to changes in the cellular mechanisms for LDL uptake or to changes in LDL production but rather to intrinsic changes in the structure and metabolism of the plasma LDL of the probucol-treated animal. These changes clearly affect uptake by pathways other than that of the classical LDL receptor but probably also affect the latter.—**Naruszewicz, M., T. E. Carew, R. C. Pittman, J. L. Witztum, and D. Steinberg.** A novel mechanism by which probucol lowers low density lipoprotein levels demonstrated in the LDL receptor-deficient rabbit. *J. Lipid Res.* 1984. **25:** 1206–1213.

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Probucol, widely used in the management of hypercholesterolemia, decreases levels both of low density lipoprotein (LDL) and high density lipoprotein (HDL) but has no consistent effect on triglyceride levels (1, 2). Patient response varies considerably but the drug is effective in a high proportion of patients, including subjects with the heterozygous or homozygous forms of familial hypercholesterolemia (HFH) (3, 4).

The mechanism by which probucol lowers lipoprotein levels remains uncertain. The drug does not appear to have a primary effect on the rate of hepatic cholesterol biosynthesis (5) but there are reports suggesting a decrease in hepatic production of lipoproteins (6, 7). The reduction in plasma HDL levels is associated with a decrease in production of apoprotein A-I (8). With regard to LDL, several investigators have reported that probucol increases its fractional catabolic rate (FCR) (8, 9) but others have found no consistent change (10, 11).

Bile acid sequestrant resins lower LDL levels by increasing hepatic LDL-receptor activity and thus increasing the rate of removal of LDL from plasma (12). Consonant with this mechanism, subjects with HFH, who genetically completely lack LDL-receptors, do not respond well to bile sequestrant therapy. The recent reports that patients with HFH respond well to treatment with probucol is therefore of considerable interest, both practically and theoretically. If an increase in LDL clearance is an important mechanism of action of probucol, the findings in HFH patients suggest that the drug may act by increasing removal via LDL-receptor *independent* pathways. The present studies were undertaken to test this hypothesis, utilizing the newly described animal model of HFH, the Watanabe Heritable Hyperlipidemic rabbit (WHHL). This inbred line lacks the high affinity LDL-receptor, has marked hypercholester-

Abbreviations: HDL, high density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; WHHL, Watanabe heritable hyperlipoproteinemic; FCR, fractional catabolic rate; HFH, heterozygous or homozygous familial hypercholesterolemia.

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olemia on a normal chow diet, and develops severe atherosclerosis (13, 14).

In this report it is shown that WHHL rabbits, like human subjects with HFH, do respond well to probucol treatment (mean decrease of 36% in LDL-cholesterol level). The LDL in the treated animals shows marked changes in composition and these are evidently associated with changes in metabolic handling of such LDL particles. From a series of kinetic studies in vivo and from studies in cultured fibroblasts, it is concluded that a major basis for the effectiveness of probucol in these animals relates to changes it brings about in the *intrinsic structure and metabolic properties of LDL.* It is these changes, leading to more rapid removal from plasma or in cell culture, rather than drug effects on the animals' mechanisms for LDL production or its tissue mechanisms for LDL removal, that appear to be the major basis for probucol action in the WHHL rabbit and also, at least to some extent, in the wild-type New Zealand White rabbit.

METHODS

Animals

Homozygous LDL receptor-deficient rabbits (WHHL rabbits) were raised in La Jolla from a mating pair provided by Dr. Y. Watanabe (13). Six WHHL rabbits aged 3 months (four female and two male) were housed in metabolic cages and maintained on standard chow diets (Universal Feed, Colton, CA) before initiating the studies. Four wild-type New Zealand White rabbits (NZW) (two male and two female) were used at 4–6 months of age.

Diets

Pure probucol (generously provided by Merrell-Dow Pharmaceuticals Inc., Indianapolis, IN) was dissolved in chloroform and added to the standard chow diet to achieve a final concentration of 1% (w/w). The $CHCl_3$ was then removed by exhaustively drying in air for 48 hr. Control chow diet was prepared using the same amount of chloroform. Plasma probucol levels were measured in three animals on drug treatment for at least 4 weeks. Plasma samples were assayed for probucol by high performance liquid chromatography after extraction with methanol–acetone utilizing a method supplied by Merrell-Dow Pharmaceutical Inc. The levels varied from 50–100 μg/ml. Plasma probucol levels in humans with familial hypercholesterolemia on conventional therapeutic doses (1 g/day) have been reported to be 20 to 90 μg/ml (3, 15).

Lipoprotein preparations and labeling

Rabbit LDL (d 1.02–1.060 g/ml) was isolated from both normal and WHHL animals by preparative ultracentrifugation, using NaBr to adjust solution densities (16).

LDL preparations were labeled with carrier-free radioiodide, ^{131}I or ^{125}I, and 1,3,4,6-tetrachloro-3,6-diphenylglycouril, a water-insoluble oxidizing agent (Iodogen, Pierce Chemical Co.) (17). Control experiments showed that LDL labeled with the Iodogen method had fractional catabolic rates (FCR) comparable to LDL labeled with the iodine monochloride method. The Iodogen method was used as it gave preparations of high specific activity with more efficient incorporation of radioiodide. The preparations were then exhaustively dialyzed and sterilized by filtration (0.45 μm filter, Millipore, Bedford, MA). Specific activities ranged from 200 to 300 cpm/ng of protein. Lipid labeling averaged 2% of total incorporated radioactivity for LDL isolated from control or probucol-treated animals. All LDL preparations were radiolabeled and used within 14 days of their initial isolation.

For lipid composition studies, plasma lipoproteins were separated by density gradient ultracentrifugation in an SW-41 rotor according to Redgrave, Roberts, and West (18). Recovery of total plasma triglycerides and cholesterol in the lipoprotein subfractions isolated through the gradient procedure was excellent (100 ± 10%).

The apoB content of LDL was measured after isopropanol precipitation of isolated lipoproteins as described by Holmquist and Carlson (19).

Turnover studies

To prevent sequestration of radioiodide resulting from lipoprotein catabolism, 3 mg of NaI was injected prior to injection of the labeled LDL. Either singly- or doubly-labeled LDL preparations (about 25–50 μCi of radioiodine and 1.5 mg of LDL protein in less than 1 ml of buffered saline) were then injected through a marginal ear vein. Samples of blood (500 μl) were obtained from different ear veins at the indicated time intervals over 25–48 hr and anticoagulated with solid EDTA. The plasmas were separated within 4 hr by centrifugation and aliquots were radioassayed using a gamma scintillation spectrometer.

Each plasma decay curve was fitted to a best-fit biexponential function using an interactive curve-peeling program on a VAX/VMS computer (Digital Equipment Corp. Marlboro, MA) as previously described (20). Fractional catabolic rates were calculated as described by Matthews (21).

Cell culture studies

Receptor-deficient rabbit skin fibroblasts (WHHL) and normal rabbit skin fibroblasts (NZW) were grown in monolayers and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells taken between the 6th and 12th passages were seeded into 35-mm tissue culture plates and used for experiments when approaching confluency (80–160 μg of cell protein/dish). Cells were preincubated for 24 hr in lipoprotein-deficient serum (6 mg of protein/ml) before initiation of uptake studies. Incubations with ^{125}I-labeled LDL were carried out for 20 hr at 37°C. After exhaustive washing with cold phosphate-buffered saline, cells were harvested by trypsinization as described previously (22). LDL degradation was measured in terms of trichloroacetic acid-soluble, iodide-free ^{125}I appearing in the medium. Iodide was precipitated from the trichloroacetic acid-soluble fraction using $AgNO_3$ (23). Acid-soluble, iodide-free radioactivity from control dishes containing no cells was subtracted from the values determined for experimental samples. Data are expressed as ng or μg of lipoprotein-protein degraded per mg of cell protein during the 20-hr incubation.

Chemical analyses

The triglyceride and cholesterol concentrations in plasma and lipoprotein fractions were determined by enzymatic methods using Bio-Dynamics/BMC Reagent Set. Protein concentrations were determined by the method of Lowry et al. (24). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of apoLDL was performed in a 4–20% gradient (25).

RESULTS

Lipid and lipoprotein responses

The responsiveness of the receptor-deficient WHHL rabbit to probucol treatment is shown in **Fig. 1.** During the first 4 weeks of treatment, the total plasma cholesterol level in the three animals in group A, receiving probucol at 1% (w/w) in their chow, dropped progressively from about 675 to about 450 mg/dl. In contrast, no change in plasma cholesterol levels occurred in the three animals in group B, receiving control chow only. The groups were switched after 4 weeks. On cessation of treatment, cholesterol levels rose progressively in group A, while the animals in group B now showed a progressive decrease on treatment, reaching a new plateau again at approximately 4 weeks. Group B was continued on probucol treatment and there was no apparent escape over an additional 5 months of treatment.

The plasma lipid and lipoprotein responses in all six animals are summarized in **Table 1.** For the group as a whole, there was a decrease in total plasma cholesterol of 23.5 ± 5.7 (range: 12.3–32.1%) after 4 weeks of probucol treatment. There was no significant change in total plasma triglycerides nor in VLDL and IDL cholesterol values. HDL cholesterol levels fell by 53%, a highly significant change. LDL cholesterol levels fell by 36%,

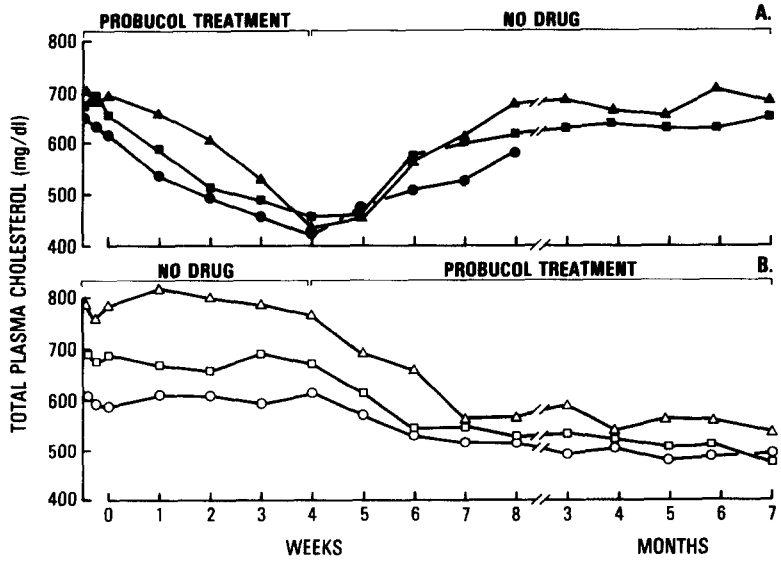

Fig. 1. Plasma cholesterol concentrations in individual WHHL rabbits before and during therapy with probucol. Panel A show results in rabbits treated with probucol (1% of chow, w/w) for the first 4 weeks and on no drug subsequently. Rabbits in Panel B were untreated for the first 4 weeks and then treated with probucol for the ensuing 6 months.

also a highly significant change. However, the concentration of apoB in the LDL fraction decreased only 9.9% and thus the cholesterol/apoB ratio fell by almost 30%. The cholesterol/triglyceride ratio in LDL also decreased, by almost 35%. SDS gel electrophoresis (4–20% gradient) of the LDL from probucol-treated and untreated animals showed no differences in apoprotein distribution (predominantly apoB with only traces of apoE and apoC). Thus, the LDL fraction from the probucol-treated animals was markedly enriched with apoB relative to lipid. Such LDL would be expected to have a greater density. Lock and coworkers (26) have observed similar trends in the LDL of probucol-treated patients.

Kinetic studies

In the first set of kinetic studies a single large batch of LDL was isolated from untreated WHHL rabbits, radioiodinated, and then injected into untreated and probucol-treated WHHL rabbits. As shown in **Table 2,** although the LDL cholesterol level was 25% lower in the probucol-treated group, there was no significant difference in the FCR of the injected LDL. The observed FCR in these receptor-deficient animals was low, as expected, being about ⅓ that seen in normal rabbits (27, 28), but it was almost exactly the same in the probucol-treated and untreated groups.

On the face of it, these results would imply that probucol treatment in these receptor-deficient animals reduced LDL levels by decreasing the production of LDL, without altering its fractional rate of removal. Such a conclusion would not necessarily conflict with reports that the FCR of LDL is increased in probucol-treated patients since those kinetic studies were done in patients having LDL receptors (8, 9). Also, in those studies the labeled LDL was in each case prepared from the patient's own plasma just before the turnover was measured. Thus, the LDL used to measure FCR on treatment was drawn from the patient taking probucol. Because the composition of LDL was different in the probucol-treated rabbits, as discussed above, and since probucol is known to be incorporated into the LDL fraction (29), we decided to test whether the LDL in probucol-treated animals might have different metabolic properties than the LDL in untreated animals. There is precedent for this in the findings of Witztum and coworkers (30) that the LDL from cholestyramine-treated animals does indeed behave metabolically quite differently from LDL isolated from the same animals not under treatment.

Three sets of experiments were carried out to compare the catabolism of LDL from untreated donor WHHL rabbits with that of LDL from probucol-treated donor WHHL rabbits. In each experiment one of the prepa-

TABLE 1. Effect of 4 weeks of probucol treatment on lipid and lipoprotein levels in six WHHL rabbits

Period	Body Weight	Plasma Triglyceride	Plasma Cholesterol	Cholesterol				LDL	
				in VLDL	in IDL	in LDL	in HDL	ApoB in LDL	Chol/ApoB
	g	*mg/dl*	*mg/dl*	*mg/dl*				*mg/dl*	
Before treatment	1566 ± 87	430.0 ± 28.5	646.6 ± 66.9	120.5 ± 27.7	101.7 ± 11.3	396 ± 61.7	8.9 ± 1.6	173.5 ± 21.0	2.27 ± 0.1	3.22 ± 0.9
After treatment	1541 ± 114	425.6 ± 9.7	493.3 ± 58.0	116.2 ± 18.4	99.2 ± 16.9	251.3 ± 38	4.1 ± 1.2	155.6 ± 14.1	1.60 ± 0.1	2.10 ± 0.4
P^a	ns	ns	<0.001	ns	ns	<0.001	<0.005	<0.005	<0.001	<0.01

[a] Student's *t*-test for paired values; ns, not significant.

TABLE 2. Comparison of LDL turnovers in probucol-treated and untreated WHHL rabbits using ^{125}I-labeled LDL prepared from pooled plasma of normal, untreated rabbits

	Untreated WHHL Rabbit (n = 3)	Probucol-Treated WHHL Rabbits (n = 3)
Total plasma cholesterol (mg/dl)	608.3 ± 34.7	543.3 ± 29.4
LDL cholesterol (mg/dl)	368.0 ± 42.1	275.5 ± 39.2
Fractional catabolic rate of pooled LDL from wild-type NZW rabbits (hr^{-1})	0.026 ± 0.002	0.023 ± 0.002

rations was labeled with ^{125}I and the other with ^{131}I so that they could be injected simultaneously into the recipients and their decay could be measured simultaneously to minimize biological variation. The donors were in every case receptor-deficient donors but the recipients were, as shown in **Table 3,** either probucol-treated WHHL rabbits, untreated WHHL rabbits, or untreated wild-type NZW rabbits. The surprising but consistent result was that the LDL from probucol-treated donor animals was cleared more rapidly than that from untreated donor animals in every case. The FCR for labeled LDL from untreated donors injected into WHHL recipients was low, as expected (0.018 to 0.027). It was not higher in the probucol-treated recipients, in agreement with the preliminary experiments (Table 2). However, when the labeled LDL had its origin in a probucol-treated donor (and was injected simultaneously) it showed a higher FCR in every case (+29 to +68%). When the same pairs of tracers were injected into NZW rabbits, the same phenomenon was again seen, i.e., the clearance of the LDL isolated from probucol-treated donors was faster than the clearance of the LDL isolated from untreated donors (+43 to +64%). Of course, the FCR values were 3 to 4 times as high in these wild-type recipients.

Examination of the LDL decay curves, whether in probucol-treated or untreated recipients, showed that the first phase of the biexponential decay was considerably greater when the labeled LDL was prepared from probucol-treated donors. A representative example is given in **Fig. 2.** The terminal slopes of the two curves were very similar. If the LDL from probucol-treated donor animals is homogeneous, then results suggest that it enters a larger extravascular pool (e.g., binds to a larger number of high or low affinity plasma membrane binding sites); if the LDL from probucol-treated donors is nonhomogeneous, the results suggest a more rapidly catabolized subclass of LDL particles.

Comparison of LDL preparations in cultured skin fibroblasts

Further evidence that the LDL in probucol-treated animals is biologically modified was obtained from studies in cell culture. The uptake and degradation of labeled LDL derived from untreated and probucol-treated WHHL rabbits was measured in cultured skin fibroblasts prepared from WHHL or wild-type NZW rabbits (**Fig. 3**). As expected, degradation of both LDL preparations by WHHL fibroblasts (Fig. 3A) was considerably slower than their degradation in the normal fibroblasts (Fig. 3B). However, both in WHHL fibroblasts and in normal fibroblasts, the uptake and degradation of the LDL from probucol-treated donors was faster than that of LDL from untreated donors. While not readily appreciated because of the low absolute values, the difference between LDL from probucol-treated donors and that from untreated donors was, in percentage terms, much greater in the receptor-deficient fibroblasts (Fig. 3A) although the absolute increment was greater in the normal fibroblasts (Fig. 3B). The curves in all cases indicated the presence of a saturable component. When a large excess of unlabeled LDL (from untreated animals) was added to compete with labeled LDL, there was clearly a saturable component both for normal LDL and for LDL from probucol-treated donors tested in normal fibroblasts (**Fig. 4**). The results suggest that at the concentrations of labeled LDL used (approximately 8 μg/ml) only about 14% of the uptake and degradation of nontreated, normal LDL by normal fibroblasts was occurring by way of nonsaturable processes, while about 40% of the degradation of the LDL from probucol-treated animals occurred via nonsaturable processes (or via pathways not competed for by native LDL).

TABLE 3. Comparison of plasma FCR for labeled LDL prepared either from untreated or from probucol-treated WHHL rabbits

Expt. #	Recipient Rabbits	Source of Labeled LDL: Untreated WHHL Rabbit	Source of Labeled LDL: Probucol-Treated WHHL Rabbit	Increase in FCR Due to Probucol
		fractional catabolic rate (hr^{-1})		
1	Probucol-treated WHHL	0.019	0.028	+47%
	Untreated WHHL	0.018	0.028	+56%
	Untreated normal NZW	0.062	0.102	+64%
2	Probucol-treated WHHL	0.019	0.032	+68%
	Untreated WHHL	0.027	0.031	+29%
	Untreated normal NZW	0.065	0.093	+43%
3	Untreated normal NZW	0.067	0.098	+46%

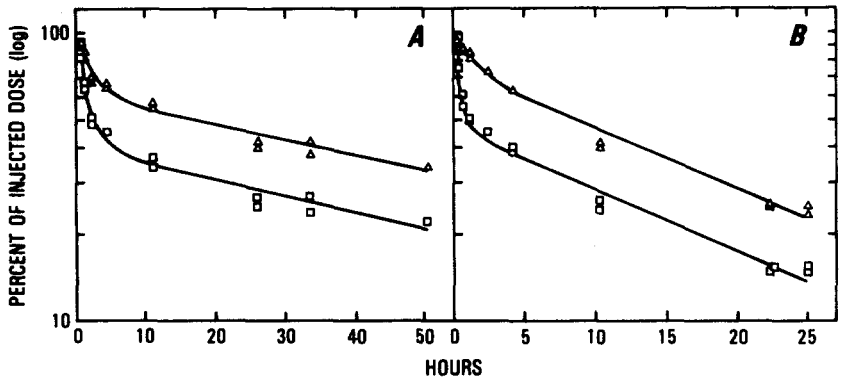

Fig. 2. Representative individual plasma decay curves for LDL prepared from a probucol-treated WHHL rabbit (□) and from an untreated WHHL rabbit (△) and injected simultaneously into a probucol-treated WHHL recipient (panel A) and into an untreated NZW rabbit (panel B). Duplicate plasma samples were analyzed at some but not all time points.

DISCUSSION

These data clearly show the high degree of responsiveness of the LDL receptor-deficient rabbit to treatment with probucol. The result is consonant with the work of others showing that LDL receptor-deficient patients also respond (3, 4). The very fact that receptor-deficient patients and animals respond as impressively as they do almost rules out a mechanism exclusively linked to induction of the LDL receptor. It could be argued that the patients and animals studied retain the ability to express at least a limited number of receptors and that treatment with probucol induces such expression. However, in these studies the FCR of normal LDL obtained from normal donor animals was equal in untreated and probucol-treated WHHL rabbits. Had the response to probucol been linked to induction of residual normal LDL receptors, the FCR of normal LDL should also have been increased in the probucol-treated rabbits.

Since the fractional catabolic rate of normal LDL was unchanged but the LDL pool size was decreased, the LDL production rate in the probucol-treated animals calculated in the conventional manner would be low.

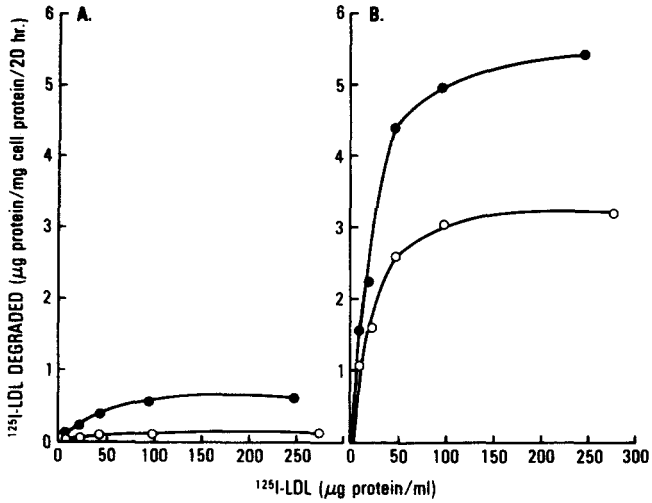

Fig. 3. Degradation of ^{125}I-labeled LDL prepared from probucol-treated (●) and untreated (○) receptor-deficient animals by (A) WHHL skin fibroblasts and (B) by normal NZW skin fibroblasts as a function of medium concentration of LDL. The cells were exposed to medium containing lipoprotein-deficient serum (5 mg of protein/ml) for 24 hr. Then the cells were incubated in fresh LDS-containing medium with the indicated concentrations of ^{125}I-labeled LDL for 20 hr and degradation was measured as described under Methods. Each point represents mean values for two separate dishes.

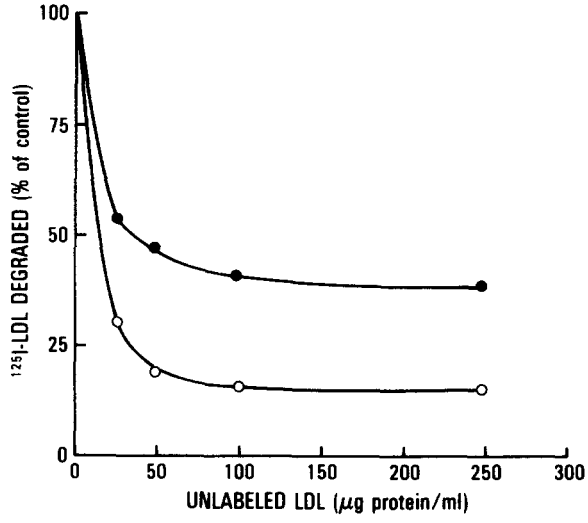

Fig. 4. Effect of unlabeled LDL from untreated WHHL rabbits on degradation of ^{125}I-labeled LDL from probucol-treated (●) and from untreated (○) WHHL rabbits in cultures of normal NZW skin fibroblasts. Incubation conditions were as described in Fig. 2 except that the concentration of ^{125}I-labeled LDL was held constant at 8.3 μg/ml for LDL from probucol-treated animals and at 7.5 μg/ml for LDL from untreated animals. In the absence of unlabeled LDL, degradation rates for LDL from treated and untreated animals, respectively, were 0.954 μg and 0.327 μg/mg of cell protein/20 hr. Each point represents the mean of duplicate incubations.

We might have concluded at that point that decreased LDL production was the basis for the effect of probucol in the WHHL rabbit. However, because an increase in the FCR of LDL had been observed in probucol-treated patients (8, 9), because of the marked changes we observed in LDL composition, because it is known that probucol and some of its metabolites are transported in LDL (29), and because of the precedent offered by the work of Witztum and co-workers (30) on changes in metabolic behavior of LDL from cholestyramine-treated animals, we proceeded to test the hypothesis that probucol-induced alterations in LDL structure in and of themselves might alter the metabolism of LDL. The results of these investigations confirmed this hypothesis and shed a different light on the mechanisms involved.

The present studies establish that LDL prepared from the plasma of a probucol-treated animal differs from normal LDL not only in composition and, presumably, structure, but also differs metabolically. The altered metabolic activity was demonstrated both in vivo and in vitro. In vivo studies showed that the FCR of the modified LDL was increased both in normal recipients and in WHHL recipients, implying an effect not limited to the high-affinity LDL receptor. If we assume that the WHHL animals are totally deficient in receptors, then the effect in these animals must be exclusively on LDL receptor-*independent* pathways. On the other hand, the increased FCR of the LDL from probucol-treated donors was also seen in wild-type NZW rabbits and the magnitude of the difference (in percentage terms) was comparable to that seen in receptor-deficient rabbits. This implies that uptake and degradation by way of the high-affinity LDL receptor may also be enhanced. This interpretation is consistent with the increased high-affinity uptake of LDL from probucol-treated donors in normal fibroblasts (Fig. 3B) and the effectiveness of native LDL in competing with LDL from probucol-treated animals for uptake in normal fibroblasts (Fig. 4).

The FCR was calculated in these studies using the method of Matthews (21) which involves analysis of the decay curve and fitting to a biexponential equation. Inspection of the decay curves showed that the percentage of injected dose disappearing rapidly during the initial phase of disappearance was greater when LDL from probucol-treated donors was used (Fig. 2) but the terminal slopes were essentially the same. In the Matthews model, the initial slope is presumed to reflect equilibration of plasma LDL with an extravascular pool of LDL. The major part of this presumed extravascular pool has been allocated to the liver (20). It may actually represent LDL adsorbed to the large surface of hepatic cell membranes, both to LDL receptors and to other binding sites on the membrane. The increase in the magnitude of the initial phase of disappearance could simply reflect an expansion of this pool of bound LDL. The other possible explanation is that there may be nonhomogeneity in the LDL from probucol-treated donors and that the first phase represents the more rapid disappearance of a subfraction. For example, most of the probucol and its metabolites in the plasma are transported in lipoproteins. Incorporation into LDL might differentially favor the removal of the larger relatively cholesterol-rich particles. This and other molecular mechanisms are currently under investigation.

These studies establish the feasibility of lowering LDL levels by inducing changes in the LDL molecule itself that cause it to be more rapidly taken up and degraded. Such a chemotherapeutic possibility has been pointed out previously (31) but not reduced to practice. It should be noted that, as with any intervention that lowers plasma LDL levels, it will be important to determine whether uptake and degradation by arterial tissue is increased also. Almost all of the interventions that have been studied work either by directly or indirectly inducing change in the rates of lipoprotein production or changes in the tissues of the treated subject that affect rates of removal from the plasma compartment. As mentioned above there is another example of drug-induced changes in LDL that affect its metabolism, namely, the finding that LDL isolated from cholestyramine-treated guinea pigs is degraded more *slowly* than native LDL both in cholestyramine-treated animals and in untreated animals (30). In that case the effect is, paradoxically, opposed to the net effect of the treatment on LDL levels. Nevertheless, it is another example of the need to consider treatment-induced changes in the metabolite itself in addition to changes in the metabolism of the treated animal or patient.

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