Low-density lipoprotein as a transporter of dolichol intermediates in the mammalian circulation

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The cholesteryl esters which make up the bulk of the core of the human low-density lipoprotein particle were removed by extraction into heptane and replaced with the fluorescent anthroyl or *N*-(7-nitrobenzyl-2-oxa-1,3-diazol-4-yl)aminohexanoyl esters of dolichol. The reconstituted low-density lipoproteins were efficiently internalized by normocholesterolaemic human fibroblasts but not by fibroblasts from patients lacking the low-density-lipoprotein receptor, or lacking the ability to internalize the receptor–lipoprotein complex. In normal fibroblasts, the reconstituted low-density lipoproteins were delivered to lyso-

somes after internalization. The results suggest that (i) dolichol intermediates in the human circulation are normally carried on low-density lipoproteins and (ii) that low-density lipoproteins are involved in the accumulation of dolichol intermediates in lysosomes during normal human aging and in certain diseases involving the lysosome. In addition, by incorporating these very hydrophobic probes into low-density lipoprotein, they can be presented to cells in culture at high concentration in a water-soluble form.

INTRODUCTION

Dolichols are linear polyisoprenoid alcohols which in humans may contain 120 or more carbon atoms. A portion of the dolichol pool is present in the form of phosphate and fatty acid esters. The phosphorylated forms are obligatory intermediates in protein *N*-glycosylation in the rough endoplasmic reticulum.

Most of the dolichol in cells, however, is present in lysosomes where it has no recognized function [1]. The ubiquitous presence of dolichol in all eukaryotic cellular and intracellular membranes suggests that additional function(s) are likely to be found.

In certain conditions, the dolichol content of tissues (lysosomes) increases dramatically without apparent biochemical/physiological explanation [2–5]. These include the process of normal aging [2], as well as the defined (aspartylglucosaminuria [3]) and probable (neuronal ceroid lipofuscinosis [4–6]) lysosomal storage diseases.

We have here attempted to obtain information on the mechanisms involved in dolichol uptake from the circulation and its delivery to, and accumulation in, lysosomes and the effect that accumulation has on normal lysosomal functions. In this paper we provide evidence that low-density lipoprotein (LDL), the major cholesterol transporter in humans, can also transport dolichol derivatives to the lysosome.

EXPERIMENTAL

Chemicals and reagents

Dolichol (C_{80-105}) from pig liver, *N*-(7-nitrobenzyl-2-oxa-1,3-diazol-4-yl)aminohexanoic acid (NBD-hexanoic acid), *n*-heptane and purified potato starch were from Sigma, St. Louis, MO, U.S.A. Anthracene-9-carboxylate and triphenylphosphine were from Aldrich, Milwaukee, WI, USA, and diethyl azodicarboxylate was from Fluka, Ronkonkoma, NY, U.S.A. The reaction solvent for synthesis of dolichyl esters was tetrahydrofuran treated with $LiAlH_4$ and distilled at 66 °C just before use. All other chemicals/solvents were either h.p.l.c. or reagent grade.

The familial hypercholesterolaemic cell lines were from the Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ, U.S.A. and had repository numbers GM00701B (FH-1) and GM02408C (FH-2).

Synthesis of fluorescent derivatives of dolichol

The synthesis of anthroyl dolichol using the chemistry of the Mitsunobu reaction [7,8] has been described [9]. More recently we have demonstrated the synthesis of NBD-(hexanoyl) dolichol using the same basic methodology [10]. Purification was by t.l.c. on plates of silica gel H (solvent hexane/diethyl ether/acetic acid, 65:35:1, by vol.) and/or h.p.l.c. on C_{18} using methanol/propan-2-ol gradient systems [9].

The purity of the synthetic esters was determined on the basis of the following. (i) A direct proportionality of the u.v.–visible signal resulting from dolichol (210 nm) with that of the fluorescent moiety carried on the backbone (254 nm for anthracene; 332 and/or 464 nm for NBD) during h.p.l.c. runs. (ii) All of the 254 nm and 332 nm/464 nm signal seen in h.p.l.c. profiles was associated with the derivatized dolichols, indicating that the preparations were free of fluorescent contaminants. In addition, the fluorescence spectra obtained for h.p.l.c.-purified anthroyl dolichol and NBD dolichol were examined in dimethyl sulphoxide and found in each case to be characteristic of the respective fluorescent substitution ([9]; L. W. Jiang and J. W. Rip, unpublished work).

Isolation of plasma lipoproteins

Plasma was collected from the heparinized blood of healthy volunteers after an overnight fast. Plasma lipoproteins were separated by discontinuous ultracentrifugation as described by Redgrave et al. [11]. The samples were spun for 24 h at 288 000 *g* at 20 °C in a Beckman SW 41 ultracentrifuge rotor. The LDL was clearly visible as an orange–yellow band between density $d = 1.006$ and $d = 1.063$ (i.e. bands II and III as indicated in ref. [11]). Pooled LDL band III from 20 ml of plasma (6 ml in

Abbreviations used: LDL, low-density lipoprotein; NBD-hexanoic, *N*-(7-nitrobenzyl-2-oxa-1,3-diazol-4-yl)aminohexanoic; r[AD]LDL, reconstituted anthroyl dolichol-containing LDL; r[NBD]LDL, reconstituted NBD-dolichol-containing LDL; nLDL, native LDL.

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volume containing about 7.5 mg of protein) was dialysed at 4 °C against two 4-litre changes (24 h each) of 0.3 mM disodium EDTA, pH 7.4.

The lower two-thirds of the delipidated plasma fraction ($d = 1.21$) was removed from each SW 41 tube, pooled and dialysed at 4 °C against two changes (24 h each) of 4 litres of 0.15 M NaCl/1 mM EDTA, pH 7.0. After dialysis, portions of LDL and delipidated plasma were assayed for total cholesterol and protein [12,13].

Preparation of LDL containing anthroyl dolichol and NBD dolichol

The basic procedures described by Gustafson [14] and Krieger et al. [15] for removing endogenous cholesteryl esters from the LDL core and replacing them with cholesteryl esters of specific composition were followed.

Dialysed LDL equivalent to 1.9 mg of protein was transferred to the bottom of a 15 ml Corex centrifuge tube containing 25 mg of purified potato starch and gently vortexed to give a uniform suspension. The mixture was frozen (as a suspension) on the sides of the lower one-third of the tube by rotating the tube quickly over a small quantity of crushed solid CO₂. Samples were then lyophilized overnight and stored until use at 4 °C under vacuum in a desiccator containing P₂O₅.

Neutral lipids were removed from the LDL–starch complex using a series of four heptane extractions (5 ml each at –10 to –20 °C) each followed by a centrifugation (480 *g*, 10 min, 4 °C). After the removal of the last heptane supernatant, 5 mg of anthroyl dolichol in 300 μ l of heptane was added per tube (i.e. 1.9 mg of protein). The mixture was carefully vortexed (to keep the suspension in the lower 2 cm of the tube) and incubated at –10 °C to –20 °C for 1.5 h. The samples were then placed on ice and freed of solvent by evaporation under nitrogen until only a dry powder remained.

The reconstituted anthroyl dolichol-containing LDL (r[AD]LDL) was hydrated by addition of 2 ml of 10 mM Tricine, pH 8.0, to each tube followed by incubation at 4 °C for 42 h. The soluble r[AD]LDL was purified away from starch and the bulk of the unincorporated anthroyl dolichol by centrifugation [480 *g* (2000 rev./min) 10 min, 4 °C]. The supernatant was carefully removed and centrifuged [17400 *g* (12000 rev./min), 20 min, 4 °C] in 1.2 ml plastic Microfuge tubes. The final supernatant (approx. 2 ml) was adjusted to the density of plasma ($d = 1.0063$) by dialysis at 4 °C against 4 litres of a solution containing NaCl (11.42 g/l) and disodium EDTA (0.10 g/l) (pH 7.0).

The dialysed r[AD]LDL was placed in the bottom of an SW41 tube adjusted to 4 ml with $d = 1.0063$ solution, and 1.3 g of KB was added [11]. A step gradient was constructed (as described earlier) and samples were spun for 24 h at 288000 *g* (41000 rev./min) at 20 °C. The upper $d = 1.006$ step was carefully removed and discarded as it contained a small quantity of free anthroyl dolichol.

The fluorescent material (visualized using a u.v. lamp) running in the $d = 1.006$ to $d = 1.063$ range (originally isolated as LDL) was removed and dialysed against 0.3 mM sodium EDTA, pH 7.4 (2 $\times$ 2 litres at 4 °C; 24 h each time). After dialysis, r[AD]LDL was concentrated in the dialysis tubing by exposure to poly-(ethylene glycol) 8000 flakes at 4 °C. The composition of r[AD]LDL with respect to protein and anthroyl dolichol was then determined, and u.v.–visible and fluorescence spectra were obtained.

NBD dolichol LDL (r[NBD]LDL) was prepared using identical procedures except that a smaller amount of NBD dolichol was available for the reconstitution.

Fibroblast uptake of r[AD]LDL and r[NBD]LDL

Fibroblast cell lines were maintained in Hams F-10 medium with 10% (v/v) fetal calf serum at 37 °C and 5% CO₂. Line DH was from a normal subject and the other two were from patients with homozygous familial hypercholesterolaemia. Line FH-1 was receptor-negative (receptor activity < 1% of normal), whereas FH-2 exhibited normal receptor binding with an internalization index of 2 (relative to 17–48 for normocholesterolaemic fibroblasts).

Confluent cells were subcultured by trypsin/EDTA treatment and plated into slant tubes containing 10.5 mm $\times$ 35 mm coverslips. Subconfluent monolayers were washed sequentially with F-10 medium lacking fetal calf serum, and PBS and placed in 1.0 ml of F-10 medium containing 5% (v/v) lipoprotein-deficient human plasma (final protein concentration 5 mg/ml) for 24 h. At this time, normal LDL, r[AD]LDL or r[NBD]LDL equivalent to 50 μ g of protein was added per culture and the cells were examined by laser-scanning confocal microscopy after an ad-

Figure 1 U.v.–visible spectra of (a) r[AD]LDL and (b) r[NBD]LDL in $d = 1.006$ solution (8.77 g/NaCl and 0.40 g/l disodium EDTA, pH 7.4) obtained in a Beckman diode array spectrophotometer

In (a) the peak values corresponding to dolichol (206 nm) and anthracene (254 nm) are indicated with arrows. The inset shows the 280–400 nm range on the same sample at greater resolution. The four inset peaks indicated by arrows are those that appear in the excitation spectrum for r[AD]LDL [Figure 2a(1)] at 460 nm emission. In (b) signals are shown for dolichol (206 nm) and NBD (332 nm and 464 nm). Again the range from about 206 nm to 600 nm is shown in expanded scale and the arrows mark the wavelengths of peak signals.

ditional 24 h. In other studies, total endocytosis of fluorescent dolichols into fibroblasts was measured as a function of time and reconstituted LDL concentration. Previous studies with anthroyl dolichol [9] showed that the fluorescent ester linkage is not hydrolysed by esterases present in fibroblasts or in the culture media.

RESULTS

Characterization of native LDL (nLDL)

The recovery of the major LDL band (band III, ref. [11]) was about 0.4 mg of protein/ml of plasma, and the ratio of cholesterol (after saponification) to protein in nLDL was 1.4. No cholesterol could be detected in the lipoprotein-deficient plasma when portions equivalent to 1 mg of protein were assayed.

Characterization of r[AD]LDL and r[NBD]LDL

The location of components carrying anthroyl dolichol in gradients was visualized using u.v. light. The r[AD]LDL ran as a fairly broad band almost entirely between the $d = 1.006$ and $d = 1.063$ interfaces. It did not extend into the $d = 1.006$ layer, the top few

Figure 2 Fluorescence spectra of (a) r[AD]LDL and (b) r[NBD]LDL taken in 0.3 mM EDTA, pH 7.4, at a protein concentration of 4 μ g/ml

In each case the solvent spectrum has been subtracted. In (a) we show for r[AD]LDL (1) the excitation spectrum for emission at 460 nm and (2) the emission spectrum for excitation at 386 nm. (b) represents for r[NBD]LDL (1) the excitation spectrum for emission at 535 nm, and (2) the emission spectrum at excitation at 478 nm.

Figure 3 Kinetics of uptake of anthroyl dolichol incorporated into LDL

Fibroblast cultures were exposed for 20 h to lipoprotein-free media and then treated as follows. (a) Supplemented with 35 μ g/ml r[AD]LDL (protein) for time periods of up to 24 h. The values were corrected for background by subtraction of fluorescence of parallel cultures labelled with nLDL. Vertical bars give the S.E.M. ($n = 3$). The 24 h point is the average of duplicate determinations. (b) Supplemented with r[AD]LDL (4–40 μ g/ml protein) and then incubated for an additional 24 h. Vertical bars give the S.E.M. The fluorescence value of 570 at LDL concentration 32 μ g/ml is the average of two values. For both (a) and (b), fibroblasts were grown in slant tubes in 1 ml of medium. Once labelling was completed, fibroblasts were washed twice with 2.5 ml of Hams F-10 medium containing 10% fetal calf serum, and (3×2.5 ml) with PBS. The cells were then solubilized in 1.0 ml of 0.1 M NaOH, and fluorescence measurements were made.

millimetres of which showed a minor fluorescent band presumed to be free anthroyl dolichol. The lower $d = 1.21$ layer after centrifugation no longer showed any fluorescence.

Of the 3.8 mg of LDL (protein) used in preparing r[AD]LDL, 1.66 mg was recovered along with 1.6 mg of anthroyl dolichol in the r[AD]LDL. The protein/dolichol ratio of r[AD]LDL was 1.05, compared with protein/cholesterol ratio of 0.72 in the starting nLDL. The recoveries of protein and anthroyl dolichol in the form of r[AD]LDL were 44% and 16% respectively. These values are similar to those obtained for LDL reconstituted with cholesteryl linoleate under the same conditions (56% protein and 24% cholesteryl linoleate [15]).

Reconstitutions with NBD dolichol were carried out at lower lipid concentration. Studies with cholesteryl linoleate have shown that the mass ratio of lipid ester to protein in LDL is increased by increasing the amount of lipid ester (relative to protein) during reconstitution [15]. Of the 1.9 mg of protein and 0.84 mg of NBD dolichol used in preparing r[NBD]LDL, 858 μ g of protein and 115 μ g of anthroyl dolichol were recovered in the reconstituted LDL. The protein/dolichol ratio in this case was much higher (i.e. 7.5) than in r[AD]LDL. Recoveries of protein and NBD dolichol in this case were 45% and 14% respectively. The u.v.-visible and fluorescence spectra for r[AD]LDL and r[NBD]LDL are shown in Figures 1 and 2 respectively. In each

Figure 4 Confocal images of fibroblast cultures exposed for 20 h to lipoprotein-free media and then supplemented with native or reconstituted LDL carrying dolichol-bound fluorescence

All images were taken under identical conditions, i.e. same pinhole size and same blackness (background) level. (a) Normal fibroblasts/r[NBD]LDL; (b) FH-1 fibroblasts/r[NBD]LDL; (c) FH-2 fibroblasts/r[NBD]LDL; (d) normal fibroblasts/nLDL; (e) FH-1 fibroblasts/nLDL; (f) normal fibroblasts/r[AD]LDL.

case the signals obtained are consistent with the composition of the reconstituted LDL.

The kinetics of uptake were studied with r[AD]LDL. Under the labelling conditions used, fluorescence resulting from endocytosis of r[AD]LDL increased in a near-linear fashion over the 24 h duration of the experiments (Figure 3a). Uptake was also directly proportional to LDL (protein) concentrations between 4 and 50 $\mu\text{g}/\text{ml}$ and above (Figure 3b; J. W. Rip, M. M. Blais and L. W. Jiang, unpublished work).

The results of confocal-microscopy studies appear in Figure 4. Normal human fibroblasts effectively internalize r[AD]LDL (Figure 4a) and r[NBD]LDL (Figure 4f), and appear to deliver these structures to the lysosome. In contrast, the reconstituted LDLs are not internalized by fibroblasts from patients with homozygous familial hypercholesterolaemia; this is shown for r[NBD]LDL (Figures 4b and 4c), and similar observations were made using r[AD]LDL (results not shown). As controls for background fluorescence, the normal and one pathological cell line were 'labelled' with nLDL (i.e. non-fluorescent; Figures 4d and 4e). These fibroblasts demonstrated zero fluorescence under the standard conditions for visualization.

To obtain additional evidence that LDL reconstituted to contain fluorescent esters of dolichol interacts with the LDL

receptor, competition experiments were carried out in which the ability of nLDL to compete with r[AD]LDL for endocytosis was assessed (Figure 5). As the amount of nLDL in a r[AD]LDL/nLDL mixture increased, the amount of r[AD]LDL endocytosed decreased dramatically. The competition observed provides additional evidence for (i) uptake via the LDL-receptor pathway and (ii) a lysosomal localization of endocytosed dolichyl esters.

Co-localization of anthroyl dolichol with fluorescein isothiocyanate-dextran 40S (a recognized lysosome marker) has previously been demonstrated [9], as has co-localization of anthroyl dolichol and β -N-acetylhexosaminidase activity in Percoll gradients of post-mitochondrial supernatants of labelled fibroblasts (J. W. Rip, M. M. Blais and L. W. Jiang, unpublished work).

DISCUSSION

The LDL particle is soluble, composed of a mainly cholesteryl ester core surrounded by a polar shell of phospholipid, unesterified cholesterol and apoprotein B [16]. Heptane extraction removes over 99% of the core cholesteryl esters and these were replaced with anthroyl dolichol or NBD dolichol. The reconstituted LDL ran with the same mobility as nLDL in lipoprotein

Figure 5 nLDL competes with r[AD]LDL for endocytosis into human fibroblasts

Fibroblasts were exposed for 20 h to lipoprotein-free medium and then supplemented for 24 h with 35 μg/ml r[AD]LDL (protein) or one of a series of mixtures of native and reconstituted LDL (each containing 35 μg/ml r[AD]LDL), in which the ratio of native to reconstituted LDL was increased. Cells were prepared for fluorescence measurements as indicated for Figure 3. Vertical bars represent the S.E.M. ($n = 3$).

gradients, although the bands were wider. This may be due to the wide size range of dolichol when compared with single homologue C_{27} cholesterol.

The studies on biological activity with normocholesterolaemic fibroblasts indicate that r[AD]LDL and r[NBD]LDL bound to the LDL receptor, and that the reconstituted LDL was taken up and delivered to the lysosome. Little or no reconstituted LDL appeared in fibroblasts from patients who lacked the LDL receptor or lacked the ability to internalize the LDL-LDL-receptor complex. Earlier studies indicated that fluorescent esters could also be taken up from liposomes and when added directly to media [9,10]. Uptake in these cases obviously involves different mechanisms. No evidence of LDL-receptor-independent uptake was seen during labelling of fibroblasts with r[AD]LDL or r[NBD]LDL, as fibroblasts lacking the LDL receptor demonstrated no fluorescence under the standardized conditions for visualization.

Our findings are significant in relation to dolichol accumulation seen in normal human aging and in diseases related to the lysosome [2–6]. The dolichol content of human tissues/lysosomes increases with age (up to 8-fold over 60 years). In addition, concentrations of dolichol, dolichyl phosphate and dolichyl pyrophosphoryl oligosaccharides are elevated in the lysosomes

of children affected with neuronal ceroid lipofuscinosis. Our studies suggest that LDL may be the transporter involved in the accumulation of these structures within the lysosome. Further studies will establish if all fatty acyl, phosphate and pyrophosphate esters, as well as the alcohol forms, can be transported or if there are specific structural determinants essential for transport on LDL.

Mammalian plasma contains 150–300 ng/ml dolichol most of which is in the high-density lipoprotein fraction [17,18]. However, to be an efficient carrier (of dolichol), there is no requirement that LDL ever accumulates significant amounts of dolichol as part of its structure.

In addition, dolichol and its derivatives are extremely hydrophobic and insoluble in aqueous media. This property makes it difficult to deliver these probes efficiently to cells and lysosomes. The LDL particle including those reconstituted with fluorescent dolichyl esters are water-soluble. This provides us with the means of targeting large quantities of fluorescent dolichol probe directly to the lysosome. The dolichol loaded lysosomes can be used to study the effect of increased dolichol content on lysosomal functions including transport, membrane fusion and enzyme catalysis.

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