Prolonged Serum Half-Life of Antineoplastic Drugs by Incorporation into the Low Density Lipoprotein

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ABSTRACT

In vitro and in vivo data have indicated that tumor cells actively internalize the low density lipoprotein (LDL) from the circulation. In order to achieve a selective delivery of drugs to tumor cells via the LDL pathway, we have incorporated oleyl derivatives of methotrexate and fluorouridine (FdUrd) into LDL particles. Three different incorporation procedures were studied: Method A, the dry film method; Method B, the transfer protein method; and Method C, the delipidation-reconstitution method. In all cases, 3H-labeled drug was incorporated into 125I-labeled LDL to yield double-labeled particles so that the behavior of both drug and carrier could be followed simultaneously. The last method led to the highest drug loading and it was possible to incorporate 50–70 molecules of dioleoyl-FdUrd per LDL particle as compared with about 18 molecules of drug when utilizing the transfer protein procedure. In vitro studies on the interaction of dioleoyl-FdUrd-LDL particles, obtained by the delipidation-reconstitution method, with the hepatocellular carcinoma cell line Hep G2, indicated that these reconstituted particles were equally effective in competing for LDL binding as native LDL. Moreover, drug delivery to Hep G2 cells occurred at the same rate as cellular association of the apolipoprotein B. In vivo studies on the fate of dioleoyl-FdUrd-LDL complexes in rats indicated that the serum decay was increased as compared with native LDL. The half-life of 6–9 min is, however, considerably prolonged as compared to the free drug (t1/2 < 1 min).

It is suggested that the 6-fold increased serum half-life of the drug-LDL complex accompanied by the possibly more specific tumor delivery may lead to an increased therapeutic effect.

INTRODUCTION

LDL1 forms a homogenous class of particles present in human blood in large quantities. One particle contains about 1500 molecules of cholesteryl esters in its core which is surrounded by 500 molecules of free cholesterol and 800 molecules of phospholipids. Regarding the substantial lipid fraction and its spherical shape, the low density lipoprotein shows some resemblance to liposomes. However, LDL is not rapidly cleared by the reticuloendothelial system and can therefore be considered as the natural equivalent of the so-called “stealth” liposomes (1, 2).

The lipid core of LDL is covered by a sole M, 514,000 protein, apoprotein B, which is responsible for recognition of the protein by a well-established specific receptor (3). Binding of LDL by the LDL receptor is followed by internalization and consequently cholesterol is provided to the cell in order to meet its requirements for building or maintaining stable membranes. The high demand for cholesterol of tumor cells may explain the high expression of LDL receptors (4–10).

About 10 years ago various authors (5, 11, 12) suggested the application of the low density lipoprotein as a carrier for antineoplastic drugs. Krieger et al. (13), Shaw et al. (14), and Rudling et al. (15) incorporated cytostatic agents into LDL in order to elicit cytotoxic effects. However, although in vitro promising data were obtained which showed that LDL could be rendered cytotoxic, no evidence was provided to show its effectiveness in vivo.

In order to incorporate antineoplastic drugs into the cholesteryl ester core of LDL, these compounds must be rendered lipophilic which may be achieved by coupling either lipophilic side chains or retinoyl (16, 17). In the present study we have coupled two oleyl chains to methotrexate or FdUrd and evaluated three incorporation procedures: Method A, the dry film method (14, 18), the passive partitioning of drug from a solid surface to the lipoprotein; Method B, a method involving enzymatic transfer by plasma transfer protein; and Method C, the solvent extraction method as described by Krieger et al. (19, 20).

Throughout our study we have labeled the LDL carrier with 125I and used 3H-labeled (pro)drugs for incorporation in the lipoprotein. This enabled us to follow the fate of both the drug and carrier simultaneously.

MATERIALS AND METHODS

Materials

125I, sodium salt (98.5% pure), was obtained from Amersham, United Kingdom. [3,3',5,7,7'-H]Methotrexate (sodium salt, 98% pure, No. TRK-224) was obtained from Amersham. 5-[6-3H]FdUrd (99.9% pure, No. NET 774) was from New England Nuclear, Boston, MA.

Human serum albumin and agarose were purchased from Sigma Chemical Co., St. Louis, MO. Bio-Gel A-50 m was from Bio-Rad Laboratories, Richmond, CA. Fetal calf serum was obtained from Boehringer Mannheim, Mannheim, Germany, and Dulbecco's modified Eagle's medium was from Flow Laboratories, Irvine, Scotland. Celite 545 was purchased from Fluka, Buchs, Switzerland. [3H]Dioleymethotrexate was synthesized by the neutral esterification of methotrexate with an alkyl halide and cesium carbonate according to the method of Rosowsky and Yu (21).

The procedure as described by Nishizawa and Casida (22) utilizing oleoyl chloride, was followed to convert [3H]FdUrd to [3H]dioleoyl-FdUrd. Full details of the syntheses of [3H]dioleoyl-MTX and [3H]-dioleoyl-FdUrd will be published elsewhere.

Lipoprotein

LDL was isolated from human plasma at density 1.024 < d < 1.055 g/ml by two repetitive centrifugations according to the method of Redgrave et al. (23) as described previously (24). The LDL preparation contained solely apolipoprotein B (99.97%) and no degradation products were noticeable when checked by electrophoresis in sodium dodecyl sulfate gels. Radioiodination of LDL was done according to the [125I]-iodine monochloride method described by Bilheimer et al. (25). Lipoprotein-deficient serum was isolated as the bottom fraction (density > 1.24 g/ml) from human plasma after centrifugation as described previously (24).

Radioactivity Analysis

Samples were assayed for radioactivity utilizing a Packard 1500 Tri-Carb liquid scintillation analyzer equipped with software validated for 3H-125I double-labeled samples.
Preparation of Drug-LDL Particles

Three different methods were used for incorporation of lipophilic prodrug into LDL. For all methods, [3H]labeled LDL was used to obtain the double-labeled drug-lipoprotein complex.

Method A (Dry Film/Celite Method). [3H]Dioleoylmethotrexate (150 µCi, 168 µg, dissolved in toluene) was added to a glass tube containing 20 mg Celite. The drug was coated on the solid surface by evaporation under N2. After complete evaporation of all solvent, 250 µl of a solution of [3H]-LDL (550 µg, in PBS-1 mM EDTA, pH 7.4) were added. The mixture was incubated under N2 while shaking continuously (overnight). The complex was then purified over a 10.0-ml Sephadex G-25 column to discard unbound drug and Celite material.

Method B (Transfer Protein Procedure). This method is basically a modification from the method previously described by Blomhoff et al. (26) and is based upon the presence of a cholesterol ester transfer protein in serum. First 50 µl (50 µg) of [3H]dioleoylmethotrexate in toluene were evaporated to dryness in a glass tube, the compound was then dissolved in 50 µl of acetone, and 500 µl of a human serum fraction (density > 1.24 g/ml) lipoprotein-deficient serum were added. After evaporation of the acetone with N2, 300 µl of PBS-1 mM EDTA were added. This lipoprotein-deficient serum-dioleoylmethotrexide preparation was then incubated with 500 µg [3H]-LDL at 37°C for 4 h. The mixture was then purified by density ultracentrifugation and gel chromatography.

Method C (Delipidation-Reconstitution Method). The lipophilic prodrugs were incorporated in LDL according to a modification of the heptane-starch method described previously by Krieger et al. (11, 20). [3H]-LDL (470 µg) was freeze-dried for 5 h with 8 mg potato starch in a siliconized tube. The neutral lipids were extracted with heptane and to the residue were added subsequently 200 µl cholesteryl oleate (= 3 mg) in heptane and 100 µl [3H]dioleoylmethotrexide (112 µg, 10.3 x 10⁶ dpm/3H) in toluene. The organic solvents were then evaporated under N2 at room temperature. The dry residue was then incubated at 4°C with 1.0 ml of a 10 mM tricine (A'-tris(hydroxymethyl)methylglycine) buffer, pH 8.4 (calbiochem), with 1 mM EDTA. After 14 h, the starch and unincorporated material were removed by centrifugation over a Sepharose column that was saturated with PBS-1 mM EDTA. Additional centrifugations were performed to discard unincorporated drug and to yield a drug-LDL complex that contained 30–70 molecules of dioleoylmethotrexide per LDL particle. For the incorporation of [3H]dioleoylmethotrexate in LDL, the same procedure as described above could be followed.

In Vivo Studies

Male Wistar rats (250–330 g) under Nembutal anesthesia were given injections of 0.5–1 ml of sample in the vena cava. At regular intervals, blood samples were taken from the vena cava at least 1 cm below the site of injection and liver lobules were excised. Blood samples were centrifuged and 200 µl of serum were mixed with 10 ml of HIONIC scintillation cocktail before assay for [3H] and [125I] radioactivity.

Liver lobules were weighed and homogenized. Homogenate (500 µl) was incubated with 750 µl of Soluene, mixed with 10 ml of HIONIC scintillation cocktail, and analyzed for [3H] and [125I] radioactivity.

Density Gradient Ultracentrifugations

Samples of double-labeled drug-LDL complexes obtained by different incorporation procedures were analyzed by ultracentrifugation. An appropriate amount of sample (200–600 µl, in PBS-1 mM EDTA) was left at room temperature in a poly allomer centrifuge tube. Thereafter, 1106 mg of solid KBr and PBS-1 mM EDTA were used for the complete dissolution of KBr to a final volume of 4.0 ml. Consecutive layers of 3.0, 2.5, and 2.5 ml of KBr solution (1.063, 1.019, and 1.0063 g/ml, respectively) were then added.

The tubes were centrifuged in a Beckman ultracentrifuge at 40,000 rpm (6 x 15 swing-out rotor) for 22 h at 4°C. By taking 500-µl samples, starting at the bottom of the tube, the gradient was subdivided according to density.

Agarose Electrophoresis

Aliquots of [3H]-[125I] drug-LDL complexes were subjected to electrophoresis in agarose gels at pH 8.8 (Tris-hippuric acid buffer). After electrophoresis, the gel was cut into segments which were then treated with 750 µl of soluene. After 20 h, 10.0 ml scintillation cocktail were added and the samples were assayed for [3H] and [125I] radioactivity.

Gel Chromatography

A small volume (100–200 µl) of [3H]-[125I] drug LDL complex was loaded on a 0.9- x 44-cm Bio-Gel A-50 m column. The sample was eluted with PBS-1 mM EDTA, pH 7.4, at a flow rate of 4 ml/h. Aliquots (500 µl) of the different fractions were analyzed for their [3H] and [125I] radioactivity.

Culturing of Hep G2 Cells

The Hep G2 cell line, derived from a human hepatocyte tumor, was obtained from Dr. B. B. Knowles (Wistar Institute of Anatomy and Physiology, Philadelphia, PA). The cells were cultured at 37°C in 25-cm² flasks (Costar) containing 0.2 ml of culture medium/cm² supplemented with 10% heat-inactivated fetal calf serum, penicillin, and streptomycin under CO₂-air (1:19). The medium was renewed twice a week.

Cellular Uptake of Drug-LDL Particles

Three–4 days before the experiment, Hep G2 cells were trypsinized and transferred to 2-cm² Costar dishes. Twenty h before the assay, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 1% human serum albumin (preincubation medium) to enhance cellular LDL receptor expression. Just prior to the experiment, the cells were washed and incubated with preincubation medium three times (15, 15, and 30 min, respectively). The Hep G2 cells were then incubated with LDL in Dulbecco’s modified Eagle’s medium-1% human serum albumin at 37°C in a humidified incubator. After 4 h the cells were cooled to 4°C and washed with PBS-2.5 mM Ca²⁺-0.2% bovine serum albumin (5x) followed by two final washes with PBS-2.5 mM Ca²⁺. For determination of cellular association of drug and carrier, the cells were dissolved in 500 µl 0.1 N NaOH and sonicated. Of this cell homogenate 350 µl were assayed for radioactivity and protein according to the method of Lowry et al. (27) with bovine serum albumin as standard.

RESULTS

Physicochemical Properties. The efficiency of incorporation of [3H]-labeled prodrug into the low density lipoprotein was evaluated by density ultracentrifugation, gel chromatography, and agarose gel electrophoresis. Construction of a double-labeled particle enabled the direct comparison between drug and carrier, and furthermore a proper estimation can be made of the incorporation efficiency and number of drug molecules per LDL particle.

Dry Film Incorporation Method. Fig. 1 shows density ultracentrifugation of particles obtained by the dry film method. The essential step of this method is the partitioning of drug from a solid surface to the lipoprotein. The contact area can be greatly enlarged by using glass beads (28) or Celite (29), the latter being used for this series of experiments. The shouldered shape of the [3H]methotrexate curve, distinct from the I-LDL peak, suggests that only a fraction of dioleoylmethotrexide is LDL associated. The position of the small shoulder, however, matches that of the carrier. When the dioleoyl-FdUrd-LDL preparation is subjected to ultracentrifugation, a uniform peak is found (Fig. 1B). However, this analysis may be indiscriminating because dioleoyl-FdUrd, in the absence of LDL, also floats at a similar density. When subjected...
to agarose electrophoresis, a distinct migration of LDL discriminates the required particle from free dioleoyl-FdUrd. It can be seen (Fig. 1C) that a substantial part of dioleoyl-FdUrd is found in a fraction different from 1-LDL. Low incorporation efficiencies did not allow the performance of measurable gel chromatography.

Enzymatic Transfer. The second incorporation procedure evaluated was the transfer protein method (26). Fig. 2A shows that unbound drug elutes directly after the void volume, while a substantial part of drug was collected in the same fractions as LDL. These fractions were pooled and subjected to density ultracentrifugation (Fig. 2A). The [3H]dioleoyl-FdUrd peak appeared at the same density as [125I]apo B while [125I]dioleoylmethotrexate was collected at significantly higher densities (Fig. 2B).

Fig. 2. Physicochemical analysis of [3H]dioleoyl-FdUrd ([3H]FUDRO)–[125I]-LDL complexes prepared by the transfer protein method. A, chromatography of [3H]dioleoyl-FdUrd ([3H]FUDRO)–[125I]-LDL prepared by the transfer protein method; B, agarose gel electrophoresis of dioleoyl-FdUrd-LDL.

Fig. 3. Density ultracentrifugation of drug-LDL complexes prepared by the transfer protein method. A, density gradient ultracentrifugation of the dioleoyl-FdUrd ([3H]FUDRO)–[125I]-LDL containing fraction that was previously collected after Bio-Gel A-50 m chromatography (Fig. 2A); B, density gradient ultracentrifugation of dioleoyl-MTX (MTXOL)–LDL prepared by the transfer protein method. NR, number.
Agarose gel electrophoresis of the dioleoyl-FdUrd-LDL particle obtained after density ultracentrifugation confirms the uniformity of the drug-LDL complex (Fig. 2B). In various preparations the incorporation of dioleoyl-FdUrd varied from 0.2 to 0.6% of the added prodrug. Similar experiments were performed with [³H]cholesteryl olate indicating an incorporation up to 70% of the added labeled substrate.

Delipidation-Reconstitution. The delipidation-reconstitution method (11, 20) involves freeze drying and heptane extraction. Both on gel chromatography and on agarose gel electrophoresis (Fig. 4, A and B), the [³H]dioleoyl-FdUrd peak coincided with the ¹²⁵I-LDL peak. Also [³H]dioleymethotrexate showed a gel chromatographic behavior similar to that of I-LDL (Fig. 4C). The incorporation efficiencies of the prodrugs varied from 15 to 30%. Protein recovery was 50 ± 15% (SD; n = 3).

In Vitro Incubations. The in vitro interaction of the drug-LDL complex was investigated with Hep G2 cells, a cell line with a well-established LDL receptor (30, 31). In order to test if the drug incorporation method affects the recognition of LDL by the LDL receptor, we compared the effect of increasing concentrations of unlabeled native and reconstituted drug-LDL complexes on the binding of I-LDL (Fig. 5A). Both the control and drug-LDL complex (delipidation-reconstitution method) are equally effective in competing with ¹²⁵I-LDL binding. Similarly, the dry film incorporation method does not affect the ability of LDL for LDL receptor recognition (Fig. 5B).

The delipidation-reconstitution method allowed labeled drug incorporation yields that were sufficient to perform in vitro drug delivery studies. Fig. 6 shows the effect of increasing LDL concentrations on the cell association of [³H]dioleoyl-FdUrd and iodinated LDL in the absence and presence of an excess unlabeled LDL (200 μg/ml). Uptake of both the drug and LDL clearly has a saturable component and the cell association of both particles is decreased by an equal level in the presence of excess LDL.

In Vivo Studies. Previous studies have shown that native LDL has a half-life in the rat of 5 to 6 h (32). The free drugs dioleoyl-FdUrd and dioleyl-MTX in an albumin-stabilized phosphate-buffered saline solution are cleared within minutes from plasma (half-lives < 1 min) and taken up primarily by the liver (Fig. 7).
LDL-ASSOCIATED ANTINEOPLASTIC DRUGS

Fig. 7. Serum decay and liver association of dioleoyl-FdUrd and LDL. Left, serum decay of dioleoyl-FdUrd (A). Also shown (■) is the serum decay of native LDL (separate rats). Data are average values of two rats; right, liver uptake of dioleoyl-FdUrd (△) and reference native LDL (■). Bars, SD.

When double-labeled [³H]FdUrd·¹²⁵I-LDL, prepared by the dry film method, was injected in the rat, a separation of the serum decay of drug and carrier was observed (Fig. 8A). Both the decay and liver uptake of drug exceed uptake of labeled LDL. The decay and liver uptake of ¹²⁵I-LDL appear to be slightly affected by exposure of LDL to the incorporation method.

Fig. 8B shows serum decay and liver uptake of [³H]dioleoyl-FdUrd·¹²⁵I-LDL particles obtained by the transfer protein method. Here, it is also noticed that the decay of drug is more rapid and the liver uptake percentual higher than for the ¹²⁵I-LDL. The serum decay as well as the liver uptake of ¹²⁵I label is essentially the same as with native LDL (Fig. 7).

The delipidation-reconstitution method is generating drug-LDL-particles with an in vivo behavior which differs from the aforementioned method (Fig. 9). Drug and carrier follow similar decay rates and reach similar liver uptake values.

As compared to the half-life of the albumin-bound drug, the half-life of the dioleymethotrexate is, however, significantly increased. A similar prolongation of half-life was also observed for dioleoyl-FdUrd. However, the decay of I-LDL appears to be strongly stimulated, mostly as a consequence of an increased liver uptake.

DISCUSSION

Three methods for the incorporation of drugs into LDL were evaluated on basis of quantitative association, in vitro modification of receptor recognition, and cell uptake and finally, most importantly, on basis of the in vivo fate of both the LDL and the drugs. [³H]MTX and [³H]FdUrd were derivatized in order to increase the affinity of the drugs for the hydrophobic core of the LDL molecule. For an adequate evaluation of the LDL particle and the drug, double-labeled drug-LDL complexes were used.

The dry film/Celite method is based upon the passive partitioning of drug from a solid surface into the LDL. The transfer protein method, described previously for the incorporation of radiolabeled cholesteryl oleate into lipoproteins (26), utilizes transfer enzyme activity present in plasma. Experimental conditions for drug incorporation with these two methods are relatively mild for the LDL particle, because either spontaneous or enzyme-facilitated drug incorporation is applied. In accordance with this assumption, in vitro receptor recognition of dry film [³H]dioleoyl-FdUrd·¹²⁵I-LDL was identical to native LDL and serum decay of the apo B moiety of this particle was also

Fig. 8. Serum decay and liver association of dioleoyl-FdUrd-LDL particles. A, serum decay (left) and liver association (right) of dioleoyl-FdUrd-LDL particles prepared by the dry film procedure. ■, ¹²⁵I-LDL; △, dioleoyl-FdUrd (n = 2). B, serum decay (left) and liver association (right) of dioleoyl-FdUrd-LDL particles prepared by the transfer protein method. ■, ¹²⁵I-LDL; △, dioleoyl-FdUrd (n = 2).

Fig. 9. Serum decay and liver association of drug-LDL complexes prepared by the delipidation-reconstitution method. A, serum decay (left) and liver uptake (right) of dioleoyl-MTX-LDL particles. ■, ¹²⁵I-LDL; △, dioleoyl-MTX. B, serum decay (left) and liver uptake (right) of dioleoyl-FdUrd-LDL particles. ■, ¹²⁵I-LDL; △, dioleoyl-FdUrd.
unchanged. The serum half-life of the drug, however, was not identical to its LDL carrier, which probably was due to the presence of non-firmly LDL-associated drug in the preparation (Fig. 1C). In the case of dioleoyl-MTX and dioleoyl-FdUrd the dry film procedure gave low incorporation values into LDL and, moreover, “incorporated” drug may not be associated with inner core of the LDL particle. Such a “loose” incorporation was found earlier with benzo(a)pyrene-LDL complexes obtained by a similar procedure (33).

The enzymatically catalyzed incorporation method (26) is unexplored for the incorporation of drugs in LDL. The method is very successful (up to 80% incorporation efficiency) in the case of incorporation of radiolabeled cholesteryl oleate. Density ultracentrifugation pointed out that dioleoyl-FdUrd can and dioleoyl-MTX cannot be incorporated into LDL with this procedure, indicating a high degree of structure specificity.

Although the serum half-life of apo B was not significantly altered by the transfer protein procedure (Fig. 8B), surprisingly liver uptake of dioleoyl-FdUrd was higher than that of LDL itself which may be due to “loose” incorporation of drugs into LDL.

Of the three methods tested, the delipidation-reconstitution procedure had the highest incorporation efficiency. Incorporation of 10–30% of the amount of added drugs were found. Using this procedure, LDL-drug complexes of 50–70 molecules of dioleoyl-FdUrd per LDL particle were produced but occasionally a drug loading up to 400 molecules could be achieved. Agarose gel electrophoresis and gel chromatography (Fig. 4, A and B) gave evidence that physicochemically well-defined particles can be obtained with this procedure. Migration of the particle in agarose gels was not significantly different than migration of native LDL. In vitro examination of the interaction of such reconstituted LDL with LDL receptors on Hep G2 cells indicated that the competition with the cell association of 125I-LDL was identical for reconstituted and native LDL. This indicates that the receptor recognition properties of apo B remained intact by the drug incorporation method. With [3H]-dioleoyl-FdUrd, 125I-LDL particles obtained by the delipidation-reconstitution procedure, a saturable component in the cell association was found and apparently equimolar uptake of the drug and particle association occurred. Furthermore, the presence of 200 μg/ml native LDL showed a substantial effect on the association of both the drug and the LDL particle. Thus it appears possible to produce LDL particles that show a receptor-dependent controlled delivery of dioleoyl-FdUrd to cells. However, in vivo, the delipidation-reconstitution method revealed particles which were cleared more rapidly from the circulation than the native particle. The liver appeared to be responsible for this increased decay. Although this behavior of LDL is undesired, it may be stated that the incorporation of dioleoyl-FdUrd into LDL led to a 6-fold retarded half-life in the blood. Although a high initial plasma concentration is not always a disadvantage for the tumor uptake in vivo, the anticipated higher LDL-mediated tumor cell uptake may lead to a considerably improved therapeutic effect.

The type of LDL modification responsible for the increased liver uptake is unclear at the moment. Modification of LDL, for instance by acetylation (26, 34) or oxidation (35, 36), leads to an increased uptake by the liver. This uptake is mediated by highly active receptors on liver endothelial and Kupffer cells (35, 37). The mechanism of this additional liver uptake is now under investigation and methods to prevent such an uptake must be further explored.

It is clear from the present results that promising physicochemical and in vitro data are not necessarily representative for similar results at the physiological level. In vivo serum decay and liver uptake studies indicated that two of the methods described in this paper did not significantly alter in vivo behavior of the carriers. However, further work on the efficiency of these methods is needed before in vivo application will be possible.

Further investigations are needed to analyze the mechanism of the induced liver uptake before adequate prevention of such modifications can be achieved. However, simultaneously clinical studies are initiated in order to test if the 6-fold increased serum half-life of the cytostatic prodrugs, accompanied by the possible specific tumor delivery, will lead to an increased therapeutic effect.

REFERENCES


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