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Low Density Lipoproteins as Drug Carriers in the Therapy of Macrophage-Associated Diseases

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Low density liporoteins (LDL) are internalised by the LDL receptor, which is expressed on the surface of eucaryotic cells. Metabolisation and chemical modification of LDLs lead to a shift in receptor affinity: modified LDLs are internalised through scavenger receptors, which are expressed on cells of the monocyte/macrophage lineage. Coupling of substances with pharmacological activity, such as azidothymidine, to LDL results in a cell specific uptake of these drugs into macrophages by the scavenger receptor. Thus, drug–LDL derivatives might work as tools for macrophage specific drug targeting. In this context, it is essential to know the drug binding capacity, the optimal derivatization conditions, and the amount of drug molecules covalently bound to the LDL particle. In this study, we compared methods for optimal derivatisation and estimation of coupling efficiency, such as semiquantitative lipoprotein gel electrophoresis, ultraviolet (UV) spectrophotometry, radiometric quantification, and specific protein hydrolysis.

Metabolism of plasma low density lipoprotein particles involves two distinct receptor pathways of internalisation: i, the LDL receptor pathway in almost all cell types of the human organism, ii, the scavenger receptor pathway in cells of the monocyte/macrophage system and the endothelium.

Native unmodified LDL binds to the LDL receptor via the apolipoprotein B100 (apoB) moiety of the LDL particle (1). Metabolisation of LDL, like glucosylation, aldehyde derivatisation and oxidation, alters the cellular specificity of LDL uptake. The internalisation of chemically modified LDL is mediated by the scavenger receptor (2). Derivatisation of the lysine residues of apoB abolishes binding of LDL to the LDL receptor and initiates the affinity to the scavenger receptor (3).

Previously we have shown that covalent binding of thymidine to LDL establishes affinity of this complex to the scavenger receptor and abolishes at the same time affinity to the LDL receptor (4). After the uptake of thymidine-LDL (T-LDL), thymidine was incorporated into the DNA of murine macrophages (P388 cells) (5).

Cells of the monocyte/macrophage lineage are susceptible to HIV infection (6). After adsorption and penetration of HIV, viral genomic RNA has to be transcribed by the viral reverse transcriptase into DNA, which is integrated as proviral DNA into the host genome. Monocytes/macrophages are found to be infected at early stages in the course of the disease and serve as reservoir for persistent productive infection. Therefore, a targeted treatment with antiviral drugs may be a promising therapeutic approach to inhibit the dissemination of HIV by chronically infected macrophages (7).

The thymidine analogues azidothymidine (AZT) and fluorothymidine (FLT), potent inhibitors of HIV replication, can also be bound covalently to LDL particles. Treatment of HIV-1 infected human macrophages with azidothymidine-LDL (AZT-LDL) and fluorothy midine-LDL (FLT-LDL) showed *in vitro* efficient antiretroviral activity (8, 9).

Thus, coupling of pharmacological active substances to LDL is a suitable tool for targeted drug delivery especially in the therapy of macrophage associated diseases. An im portant presupposition to use LDL as drug transporters are optimized methods for covalent coupling of drugs to LDL and determination of the degree of derivatisation. In this study, we compare four methods for the evaluation of coupling efficiency: UV spectrophotometry, lipoprotein gel electrophoresis, radiometric quantification and comparative protein hydrolysis before and after derivatisation.

MATERIALS AND METHODS

Preparation of low density lipoproteins. Preparation of LDL was performed using plasma obtained from healthy blood donors by KBr

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Abbreviations used: LDL, low density lipoprotein; UV, ultraviolet; apoB, apolipoprotein B100; AZT, azidothymidine; DNFB, 2.4-dinitrofluorobenzene.

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density gradient centrifugation. LDL fractions (d = 1.019 − 1.063 kg/l) were dialysed against standard buffer (0.15 mol/l NaCl, 0.5 mmol/l EDTA, pH 7.4, 4°C) (4).

Covalent coupling of ^{3}H-thymidine to LDL. Step I: 4-nitrophenyl-6-^{3}H-thymidine-carbonate was prepared from 70 mg 4-nitrophenyl-chloroformic acid (Sigma) and 50 mg 6-^{3}H-thymidine (20 μCi) by 60 min incubation in 20 ml dry dioxane with 2 mg dimethylaminopyridine at 80°C (N_2-atmosphere), evaporating in 2 ml methanol/acetone (1:1) and purification over a silicagel G60 column. Step II: Coupling of 4-nitrophenyl-6-^{3}H-thymidine-carbonate to the apoB moiety of LDL comprises stepwise addition of 10 mg 4-nitrophenyl-6-^{3}H-thymidine-carbonate (in 125 μl) methanol) to LDL (10 mg LDL-protein) in 1 ml phosphate buffer (250 mM, pH 10.0) and 30 min stirring at 4°C. 6-^{3}H-thymidine-LDL was purified by sephadex gel chromatography (Sephadex G75). Specific activities were obtained after liquid szintillation counting and estimation of the protein content of the reaction products.

Lipoprotein gel electrophoresis. Agarose lipoprotein electrophoresis was conducted with the Paragon LIPO Kit (Beckman) according to manufacturer's instructions. Coupling degrees obtained from UV spectrophotometry and specific activity were plotted versus electrophoretic mobility of derivatised LDL compared to native LDL. Linear regression results in a standard curve which can be used for the estimation of coupling efficiency following log(y) = 0.053 x + 1.533 [with x = coupling efficiency (moles derivate / mole LDL) and y = distance (in mm) of native LDL to derivatised LDL under standard running conditions (Beckman Paragon electrophoresis system, 100V, 30 min)].

UV spectrophotometry. ^{3}H-T-LDL was diluted to 0.02 mg protein / ml. Relative absorbance at 266 nm (λmax for thymidine) was measured with a Kontron UV spectrophotometer using native LDL (0.02 mg protein / ml) as background reference. Estimation of absorbance to thymidine ratio results in: 1 A_{266} is equal to 114 μM thymidine. These values are in good agreement with the literature [1 A_{266} = 106 μM thymidine-triphoshate (10)] and were used to calculate the degree of thymidine coupled to LDL after spectrophotometric measurement of UV absorption.

Protein hydrolysis. Analysis of derivatised LDL was done using 2.4-dinitrofluorobenzene (DNFB) (11). Treatment with DNFB converts unmodified lysine residues of apoB into acid stable dinitrophenyl derivatives. Acid hydrolysis converts acetylated, thymidilated and other less stable derivatised lysine residues of apoB back to free lysine molecules, which can be quantitated by amino acid analysis using fluorescence HPLC technique as described (12).

TABLE 1

Estimation of Coupling Efficiency
Using Specific Radioactivity

Coupling assay	Specific activity (Step I) (mCi/mmole)	Specific activity (Step II) (mCi/mmole)	Moles ^{3}H-thymidine/ mole LDL
A	15	1408	94
B	15.6	3333	214
C	20.5	7118	347

Note. The amount of ^{3}H-thymidine coupled to LDL is equal to the quotient of specific radioactivity of 6-^{3}H-thymidine-LDL (obtained after reaction step II) and 4-nitrophenyl-6-^{3}H-thymidine-carbonate (obtained after reaction step I). Both reaction products were gel purified to eliminate unbound reaction products.

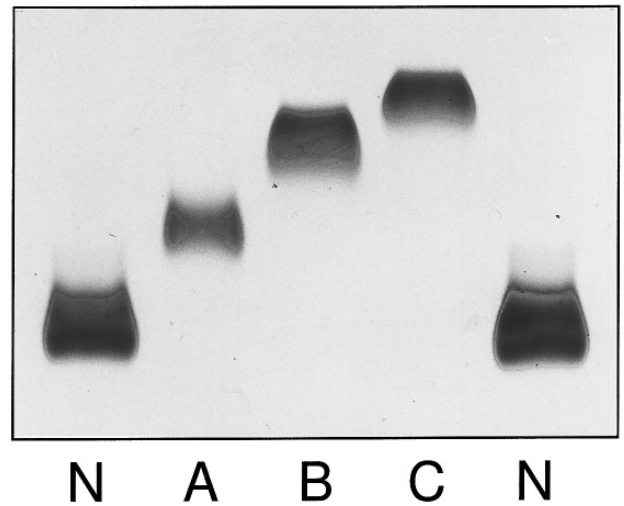

FIG. 1. Lipoprotein gel electrophoresis of LDL coupled with different amounts of ^{3}H-thymidine. Lanes **N,** native LDL; lanes **A, B,** and **C,** ^{3}H-thymidine bound LDL using a low (**A**), medium (**B**), or high (**C**) stochiometric ratio.

RESULTS AND DISCUSSION

To confirm coupling efficiency and to evaluate the degree of coupling, we coupled ^{3}H-thymidine covalently to low density lipoproteins as described before. To count the number of thymidine molecules bound to LDL molecules, we compared four different methods:

i. Measurement of radioactivity emitted by the ^{3}H-Thymidin-LDL complex. Using this approach, the number of thymidine molecules coupled to a LDL molecule can be achieved by comparison of the specific activity of the uncoupled ^{3}H-thymidine with the specific activity of the ^{3}H-Thymidin-LDL complex (Table 1).

ii. Lipid gel electrophoresis. Low density lipoproteins contain a single 550.000 D protein called apolipoprotein B100 (apoB). The amino acid sequence of apoB

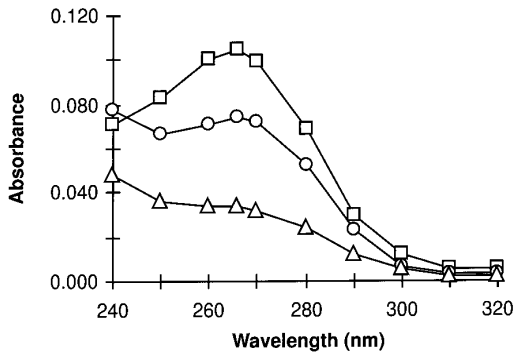

FIG. 2. Estimation of ^{3}H-thymidine coupled to LDL by UV spectrophotometry. Low (**A,** triangles), medium (**B,** circles), and high (**C,** squares) coupling efficiency corresponds to 99, 214, and 310 moles ^{3}H-thymidine per mole LDL, respectively.

TABLE 2

Estimation of Coupling Efficiency Using UV Spectrophotometry

Coupling assay	Relative absorbance (266 nm)	Thymidine μM[a]	×200 (dilution factor)[b]	LDL μM[c]	Moles thymidine/ mole LDL
A	0.034	3.9	780	7.87	99
B	0.074	8.4	1680	7.87	214
C	0.107	12.2	2440	7.87	310

Note. ^{3}H-thymidine was coupled to LDL in different stochiometric ratios (A, B, C). Absorbance was measured at 266 nm with equal amounts of underivatised LDL as background reference.

[a] 1 A_{266} is equal to 114 μM thymidine.

[b] Dilution is necessary for linear range spectrophotometry.

[c] Calculated after protein estimation of apoB.

includes 360 lysine molecules which are positively charged. Coupling of thymidine molecules to the lysine residues of the apoB results in a change of electronegativity. This change can be visualised by an altered mobility in gel electrophoresis of modified LDLs compared to native LDLs.

iii. UV spectrophotometry. Nucleosides show a characteristic absorption maximum in the ultraviolet spectrum. The number of nucleosides, which are bound to a LDL molecule can be estimated by measuring the absorbance of the nucleoside-LDL complex and subtraction of the background absorbance of the pure LDL.

iv. Amino acid analysis. The extend of modification at the lysine residues can be determined by an indirect procedure (11). Treatment with dinitrofluorobenzene (DNFB) converts unmodified lysine residues into acid stable dinitrophenyl derivatives. Acid hydrolysis of apoB converts acetylated or thymidilated lysine residues but not DNFB-lysine back to free lysine, which can be quantitated by amino acid analysis using fluorescence HPLC (12). Thus, this method enables discrimination between modified and unmodified lysine residues.

We coupled different amounts of ^{3}H-thymidine to low density lipoproteins and evaluated the coupling efficiency by each of the first three methods.

Figure 1 shows the result of three independent coupling assays (A, B, C). Due to change in electronegativity, the electrophoretic mobility of the T-LDL complex is altered in agarose lipoprotein gel electrophoresis. The pattern in Figure 1 leads to the conclusion, that complexes with higher T:LDL ratios move faster than those with lower ratios.

These results could be confirmed by estimating the specific radioactivity of the T-LDL complex. As shown in Table 1, the specific activity of ^{3}H-tymidine-LDL derivatives is different in coupling assay A, B and C and corresponds to the altered electrophoretic mobility. The number of thymidine molecules coupled to the apoB

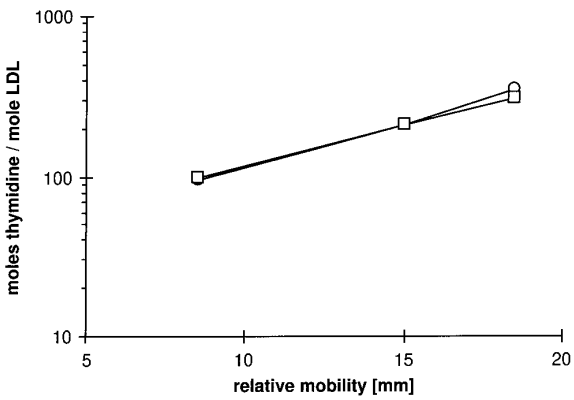

FIG. 3. Comparison of specific radioactivity and UV absorption of LDL bound ^{3}H-thymidine. Data obtained from Tables 1 (specific radioactivity, circles) and 2 (UV absorption, squares) were plotted against the electrophoretic mobility of the ^{3}H-thymidine-LDL particles in lipoprotein gel electrophoresis.

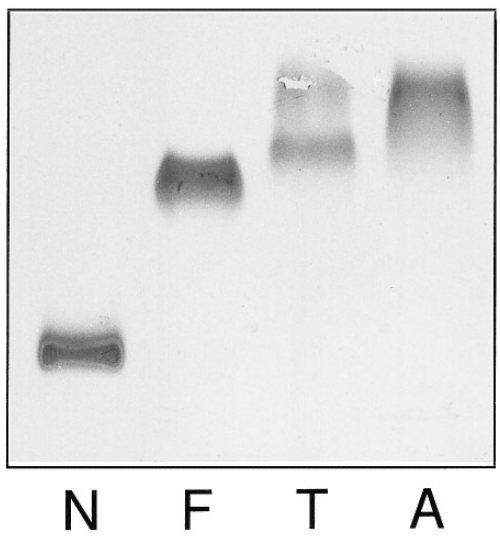

FIG. 4. Lipoprotein electrophoresis of modified LDL used for protein hydrolysis experiments. Lane **N,** native LDL; lane **F,** fluorothymidine-LDL; lane **T,** thymidine-LDL; lane **A,** acetylated LDL.

moiety of the LDL particle can be easily calculated by dividing the specific activities resulting from coupling step II through those resulting from step I.

Calculation of the thymidine:LDL ratio is also possible after UV spectrophotometric analysis of the T-LDL complexes using native LDL as a background reference (Figure 2). Wavelength scanning of all three coupling products with native LDL as background reference results in an absorption maximum at 266 nm. This maximum corresponds with the absorption maximum of free thymidine. Relative absorbance of T-LDL products at 266 nm can therefore be converted into thymidine:LDL coupling ratios (Table 2).

Both methods, UV spectrophotometry and evaluation of specific activities, show only minor differences when using the same probes for estimating the T:LDL ratio. The values obtained by each of the two methods can be plotted versus the electrophoretic mobility of the corresponding T-LDL complex in agarose gel electrophoresis (Figure 3). Relative to the electrophoretic mobility of native LDL, the three T-LDL complexes are running in a logarithmic range. Lipoprotein gel electrophoresis therefore can be used as a quick and simple method for estimating the ratio of nucleosides or other components that alter electronegativity (Figure 1).

Taken into one account, the three methods (radiometric quantification, UV spectrophotometry and lipoprotein gel electrophoresis) keep in line and can serve as a quality and quantity control for coupling efficiency in derivatisation of low density lipoproteins.

In order to apply the method of Weisgraber *et al.* (11) to determine the amount of derivatised lysine molecules using the DNFB technique, we coupled acetoacetate, thymidine and its analogue fluorothymidine covalently to low density lipoproteins. After treatment with DNFB, acid protein hydrolysis was performed. Thus, DNFB derivatised lysine residues are transformed into acid stable free DNFB-lysine, whereas acetylated or thymidilated lysine residues are hydrolysed to free lysine molecules. By comparing the lysine amounts of derivatised and native apoB , the amount of aceto- or thymidine-lysine can be calculated. Comparison with native apoB is necessary due to underivatible lysine residues, which are located inside the LDL particle and are therefore not susceptible to derivatisation.

Figure 4 shows the electrophoretic mobility of the reaction products in agarose lipoprotein electrophoresis. According to the standard curve evaluated from Figure 3, the degree of derivatisation can be estimated and is shown in Table 3. The results obtained from amino acid analysis using DNFB are in an acceptable agreement with respect to the acetylated LDL. 91 % of the derivatised residues could be detected with this method (Table 3). However, in the case of T-LDL and FLT-LDL, the results differ. Compared to the electrophoresis, only 70 % and 71 %, respectively, could be detected after DNFB treatment, indicating, that this method is less valuable for the estimation of nucleoside coupling efficiency.

TABLE 3

Estimation of Coupling Efficiency Using Protein Hydrolysis and Lipoprotein Gel Electrophoresis

Derivate	Moles derivate/ mole LDL (A)	Moles derivate/ mole LDL (B)[a]	Ratio A:B
Fluorothymidine-LDL	140	196	71%
Thymidine-LDL	170	242	70%
Acetoacetylated LDL	272	299	91%

Note. The degree of LDL derivatisation after acetylation and thymidilation detected by protein hydrolysis (A) compared to the results of lipoprotein gel electrophoresis (B) is given as a percentage (A:B).
[a] Using linear regression from standard curve in Figure 3.

However, a combination of at least UV spectrophotometric analysis and lipoprotein gel electrophoresis gives proper results concerning the degree of LDL derivatisation and is therefore suitable for estimating the drug content of a nucleoside-LDL complex.

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