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A low density lipoprotein – methotrexate covalent complex and its activity against L1210 cells in vitro

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Summary. Low-density lipoprotein particles are potential drug carriers, but only lipophilic drug species partition into the core of the system. In this paper the polar drug methotrexate has been coupled to the exterior protein of low density lipoprotein (LDL) particles using the reagent 1-ethyl-3(3′-dimethylaminopropyl) carbodiimide HCl. The coupled system was sized by photon correlation spectroscopy and the in vitro activity of the complex determined against L1210 cells maintained in medium supplemented with fetal calf serum. The reaction between methotrexate and low density lipoprotein is variable but quantifiable, about ten drug molecules being attached to each LDL particle, resulting in an increase in the radius and polydispersity of the particles. The activity of the complex against L1210 murine leukaemia cells has been demonstrated in vitro, but it is 30 times less active than free drug.

Introduction

The clinical use of antineoplastic agents is often limited by their inability to discriminate between normal and neoplastic cells. Several attempts have been made to improve the selectivity of antineoplastic agents by linking them to a carrier moiety [10], e.g., proteins [21], antibodies [25], and hormones [27], or by enclosing them in liposomes [1] or nanospheres [29]. So far no carrier system has proved to be of clinical value, and many problems remain to be tackled if this technique is to become useful, for example: (a) targeting specificity for tumour cells in vivo; (b) stability of the carrier complex in vivo; (c) release of the active agent at the target site; (d) rapid removal of exogenous materials by cells of the reticuloendothelial system; and (e) immunological reactions to the exogenous carrier. One method of circumventing some of these problems would be to use an endogenous carrier with well-defined properties capable of providing some form of tumour specificity. One such carrier that has recently stimulated interest is low density lipoprotein (LDL) [5], a component of plasma whose physiological function is the transport of cholesterol. LDL exists as a spherical particle (diameter 22 nm) with a lipid core (triglycerides and cholesterol esters) surrounded by a monolayer of cholesterol, phospholipids and protein (apoprotein B) [7]. LDL is taken up by cells through a receptor-dependent pathway, whereupon the particle is enzymatically degraded and the cholesterol utilized for cell membrane synthesis. Cells which are therefore undergoing active cell division show a greater LDL receptor activity than non-dividing cells [14]. Gal et al [8, 9] have demonstrated in tissue culture that replicating neoplastic cells metabolise LDL at higher rates than non-neoplastic cells; for example, epidermoid cervical carcinoma (EC-50) cells, metabolise LDL at a rate 20 times faster than cervical fibroblasts. On the basis of these findings, they suggest that LDL may be useful as a selective drug carrier for neoplastic cells. This work has been carried a stage further by Welsh et al [28], who have shown in vivo that a solid MAC 13 tumour in NMRI mice exhibits active LDL receptor-mediated uptake greater than that found in normal tissue.

Attempts to utilise this pathway have been fairly limited until recently, but Mosley et al. [19] demonstrated that LDL loaded with a pyrene derivative of cholesteryl oleate was able to photosensitise cells (to UV light) expressing LDL receptor activity, whereas cells lacking LDL receptor activity were not photosensitised. Rudling et al. reported last year on the incorporation of aclarubicin into LDL and the subsequent use in vitro of the LDL: aclarubicin complex against a human glioma cell line [24], finding that the complex would inhibit cell growth if the glioma cells exhibited LDL receptor activity. However, there also appeared to be some form of non-specific drug uptake as inhibition of LDL uptake did not completely abolish aclarubicin accumulation in the cells. Krieger et al. [13] have reported the inclusion of "dioleyl methotrexate" in LDL by a reconstitution method, but apart from this statement no data are presented on the final properties or use of these laoded LDL particles.

In this study we have investigated the covalent linkage of methotrexate to the surface of LDL, and the subsequent use of these coupled LDL particles in a simple tissue culture system.

Materials and methods

General reagents. Buffer salts and other reagents were of analar grade and were purchased from BDH; 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide HCl, was also purchased from BDH, and used as received. Methotrexate was a gift from Lederle Laboratories, and was used without further purification. Phosphate-buffered saline consist-

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ed of NaCl 137 m*M*, KCl 3 m*M*, Na_2HPO_4 8 m*M*, KH_2PO_4 1.5 m*M*, pH 7.4.

Extraction of human low-density liporprotein. Human plasma was isolated from venous blood (100 ml) drawn from normolipidaemic adults after overnight fasting, and its density adjusted to 1.3 g/ml by the addition of solid NaBr. LDL was extracted by rate zonal ultracentrifugation using a linear NaBr gradient [22] from density 1.3 g/ml to 1.0 g/ml formed in an MSE B14 titanium zonal rotor on an MSE Superspeed 75 centrifuge at a loading speed of 3000 rpm. The density-adjusted plasma was pumped into the bottom of the gradient (1.3 g/ml) and the centrifuge run at 45 000 rpm for 1 h 55 min at 10 °C, after which it was slowed down to the loading speed and fractions (10 ml) collected by pumping in a NaBr solution at a density of 1.3 g/ml. LDL is usually isolated in fractions 15–20, which are combined and the crude product of which is ultrafiltered (Diaflo Type XM100A membrane, Amicon Corp.) to dilute the NaBr (1 : 10 000) and replace it with phosphate-buffered saline (pH 7.4). Samples were concentrated to a final volume of approximately 10 ml, sterilised by filtration (0.22 μm), and packed aseptically under N_2, then stored at 4 °C and used within 12 days. The time for extraction to packing was usually just under 2 days, with all procedures being carried out at room temperature.

Analyis of LDL samples. LDL was quantified by measurement for apolipoprotein B (LDL = 5× protein concentration) using a modified Lowry method [16], in which 0.1% $^{w}/_{v}$ SDS is included in the copper tartrate and alkali solutions [6], with BSA as a standard. These results were in agreement with those obtained by refractive index measurements (refractive index increment 1.71×10^{-4} l/g [2]) using a Rayleigh interference refractometer (Hilger and Watts, Type M154) with 1 cm cells and a white light source.

A UV/VIS, spectrum of each sample was obtained in phosphate-buffered saline, with accurate measurements taken at 376 nm and 487 nm for use in the coupling experiments.

Photon correlation spectroscopy (PCS). A photon correlation spectrometer (Malvern Instruments, Model 4300) with 48 channels, and later a Type 7027 (Malvern Instruments) with 60 channels, were used in conjunction with a He/Cd laser (Linconix) operating at 441.6 nm with a power output of approximately 10 mW. All samples were measured after temperature equilibration at 25 ± 0.1 °C and at an angle of 90° to the incident beam.

Analysis of PCS data. For a monodisperse system of small particles the experimental data obtained are the second-order autocorrelation function $g^{(2)}(t)$ and are related to the diffusion coefficient (D) by the relationship:

$$g^{(2)}(t) = 1 + e^{-2DK^2} \cdot t,$$

where $K = (4\pi n/\lambda) \sin(\theta/2)$, n is the refractive index of the solvent, λ is the wavelength of the laser, θ is the measurement angle, and t is the delay time. Thus $[g^{(2)}(t) - 1]$ is an exponential curve and $\ln[g^{(2)}(t) - 1]$ is a straight line with a slope of $(-2DK^2)$, from which D is readily obtained. In polydisperse systems a family of exponentials will be obtained and plots of $\ln [g^{(2)} - 1]$ vs t are fitted to quadratic functions which provide the coefficient of t which is equal to the Z average diffusion coefficient [12]. The Z average diffusion coefficient is defined as $D_z = \Sigma n_i M_i^2 D_i / \Sigma n_i M_i^2$, n_i being the number of particles of molecular mass, M_i, with diffusion coefficient D_i, and from this was calculated the equivalent spherical hydrodynamic radius, assuming that the Stokes-Einstein equation applies. The normalized coefficient t^2 gives the width of distribution expressed as the normalized variance of distribution, which is defined as $2K_2/K_1^2$, where K_1 is the coefficient of t and K_2 is the coefficient of t^2; values of NVD > 0.1 indicate an almost monodisperse system [23], although this is an arbitrary demarcation.

Coupling of methotrexate to LDL. To an aliquot of LDL solution we added methotrexate and 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide HCl, both dissolved in PBS, and if necessary PBS to give the desired volume. The solution was mixed and allowed to stand at room temperature in the dark for the allotted time, after which the LDL was separated by the following methods. (See Table 1 for molar quantities.)

Separation of LDL from coupling mixture. (1) Ultrafiltration: LDL was separated by ultrafiltration (Diaflo Type XM 100A membrane, Amicon Corp.,) (1 : 10 000 dilution) with PBS, and no free methotrexate could be detected in

Table 1. Coupling of methotrexate to LDL

Experiment	mol[a] LDL10^{-9} ml^{-1}	mol MTX10^{-7} ml^{-1}	mol EDCI10^{-6} ml^{-1}	Final volume (ml)	Reaction time (h)	Method of separation	MTX coupled mol/mol	PCS r(nm) coupled	% Increase in r	NVD coupled	% Increase in NVD
1	2.0	9.0	1.3	4	3	U	41.4	13.1	11	0.1	25
2	2.0	18.0	2.6	4	3	U	Complete aggregation during reaction				
3	2.0	9.0	1.3	6	4	U	12.8	16.8	22	0.15	40
4	0.76	8.0	1.8	7	4	U	Material aggregated during ultrafiltration				
5	2.0	9.0	2.0	2	3.5	U	–	9.8	−29	0.22	39
6	1.2	5.0	2.3	6.5	5	U	8.4	15.5	21	0.17	70
7	1.5	7.0	3.0	6	5	GF	8.7	24.7	93	0.22	120
8	0.36	2.0	0.7	10	4.5	GF	ND	22.7	31	0.16	23

MTX, Methotrexate; EDC1, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide HCl; ND no MTX detected; U, ultrafiltration; GF, gel filtration chromatography

[a] Based on LDL molecular weight of 2.5×10^6

the final ultrafiltrate sample when assayed by UV analysis (376 nm). (2) Gel Filtration: The reaction mixture was concentrated by ultrafiltration to approximately 0.5 ml and passed down a Sephadex G10 column (2.2 × 10 cm) eluted with PBS. LDL was reovered in fractions 3, 4, and 5 (4 ml fractions) with unreacted methotrexate in fractions 10–14, which provided almost complete recovery of the methotrexate, with no methotrexate detectable in fractions 6–9 (when assayed by UV analysis, 376 nm). The LDL fractions were pooled and concentrated by ultrafiltration.

Both methods allowed approximately 98% recovery of the added methotrexate. After separation all LDL samples were sterilised by filtration (0.22 μm) and packed aspectically under N_2 then stored at 4 °C.

Assay for coupled methotrexate. UV/VIS absorbance measurements of the coupled material were taken at 376 nm and 487 nm in phosphate-buffered saline. The absorbance data obtained on the native uncoupled LDL at 487 nm and 376 nm and the absorbance of the coupled material at 487 nm (a wavelength at which methotrexate does not absorb) were used to calculate a theoretical absorbance for the coupled material at 376 nm due to LDL. The difference between the theoretical and the actual readings was taken to be attributable to methotrexate, and the concentration was calculated with reference to methotrexate standards.

Tissue culture. L1210 cells were purchased from Flow Laboratories, Irvine, Scotland and maintained in RPMI 1640 medium supplemented with 10% $^{v}/v$ fetal calf serum and containing penicillin 100 IU ml^{-1}, streptomycin 100 μg ml^{-1}, and 20 m*M* HEPES. Cells were grown normally in Nunclon flasks and experiments performed in Linbro multiwell (24-well) dishes, five 2-ml wells being used at each concentration. Experiments were carried out by harvesting cells in the exponential growth phase, adjusting to the starting cell concentration (2.5×10^5 cells/ml), adding the material under test, then plating out into the multiwell dishes. Two wells were sampled every 24 h and counted after dilution with phosphate-buffered saline in a Coulter Counter (Model Z_B, 100 μm counting tube); the result is expressed as the mean value of the cell counts (4/well) from both wells.

Results

The isolated LDL (2 donors), when sized by photon correlation spectroscopy, had a diameter in agreement with previous data [16, 20], having a Stokes' radius ranging from 11.8 nm to 13.8 nm and NVD (polydispersity) values between 0.06 and 0.16, indicating a slight degree of polydispersity in some samples.

Coupling of methotrexate to LDL

The method so far developed has not proved to be predictable, but it is quantitative (Table 1). In all, eight attempts (Table 1) were made to couple methotrexate to the exterior of LDL, only four of which were successful; in the remaining experiments aggregation of the LDL posed a serious problem. The initial coupling concentrations have already been successfully used to link methotrexate to bovine serum albumin [18], and the first attempt proved effective; however, to increase the quantity of methotrexate coupled the concentrations of methotrexate and coupling agent were doubled in the next attempt. At this concentration level there were 900 and 1300 molecules of methotrexate and coupling reagent, respectively, per LDL particle. This unfortunately resulted in the complete aggregation of the sample. In attempt 3 revision to original conditions again proved successful, but the quantity of methotrexate attached was approximately three times lower. In experiment 4 the levels of methotrexate and coupling reagent were raised to 1050 and 2400 molecules per LDL particle, but this again resulted in aggregation. Addition of bovine serum albumin (0.25% $^{w}/v$) to increase the physical stability failed, as the measured photon correlation spectroscopy radius was reduced to a size below that expected of LDL after the removal of residual bovine serum albumin by selective ultrafiltration (XM 100A membrane). In the next two experiments the ratios of methotrexate and coupling reagent to each particle were kept at approximately 460 and 2000 respectively, resulting in reproducible coupling. Unfortunately, in the last experiment there was no detectable methotrexate attachment, but this sample of LDL was abnormal in that its initial size was larger than expected.

Photon correlation spectroscopy on coupled LDL

In all the experiments the major effect of coupling was to produce an increase in the measured particle size (Table 1). The measured size increases range from 11% to 93% of the radius of the native LDL, and alongside this are comparative increases in the measured polydispersity (NVD) of the samples, the higher the percentage increase in radius the higher being the increase in NVD. This effect appears to be linear, as a plot of the percentage increase in NVD against the percentage increase in radius when treated statistically for the best-fitting straight line gives $Y = 17.68 + 1.06\ X$, $R = 0.86196$, which is significant at the 5% level.

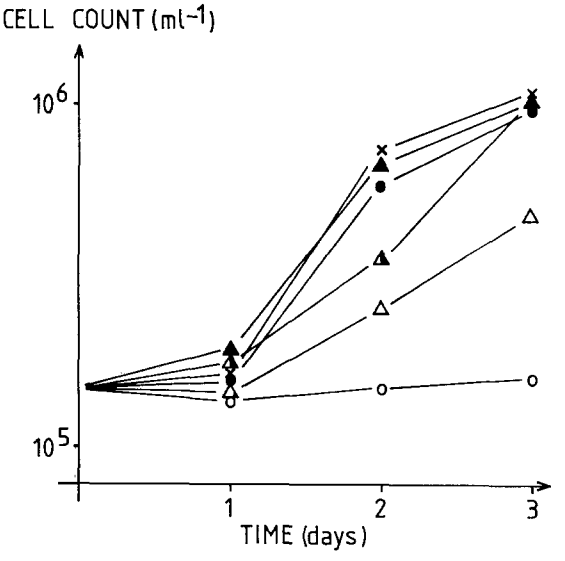

Fig. 1. Inhibition of L1210 growth in vitro. (Concentrations of MTX-LDL conjugates refer to the equivalent concentration of MTX used.) ×, control; ○, MTX 10^{-7} *M*; ●, MTX 10^{-8} *M*; △, LDL 10^{-6} *M*; , LDL 2×10^{-7} *M*; ▲, LDL 10^{-8} *M*

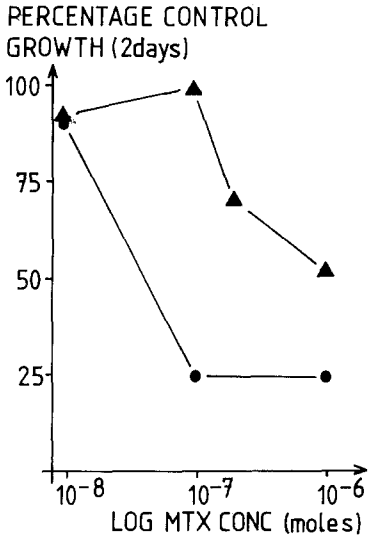

Fig. 2. Dose – response curve of L1210 to MTX and MTX-LDL conjugates (control growth at 48 h = 100%). ●, MTX; ▲, LDL

L1210 growth inhibition

The methotrexate LDL conjugates retain some biological activity against the L1210 murine leukaemia in vitro (Fig. 1). This activity is lower, however, than that of free methotrexate (Fig. 2). Methotrexate at a concentration of $10^{-7}M$ completely inhibitis growth, but at 10^{-8} *M* it has no effect at all. The methotrexate LDL conjugates do not affect cell growth at equivalent methotrexate concentrations of 10^{-8} *M* and 10^{-7} *M*. Only at the 2×10^{-7} *M* level does growth inhibition become noticeable, and when the concentration is increased to 10^{-6} *M* there is a definite effect on cell growth. The measured inhibition at 10^{-6} *M* is less than that of free methotrexate at 10^{-7} *M*, however, so that the activity of the bound methotrexate has been reduced overall by a factor of about 30 (Fig. 2).

Discussion

The data presented here indicate that methotrexate can be covalently linked to the exterior of low-density lipoprotein by using a carbodiimide coupling agent, and that the coupled methotrexate retains some degree of biological activity.

About 10 molecules of methotrexate can be attached to each LDL particle, a result which can be compared to that obtained in a similar experiment for covalent binding of methotrexate to bovine serum albumin [11], which resulted in 14–15 molecules of methotexate being bound per molecule of bovine serum albumin. As linkage by this method is most likely to occur via lysine groups on both molecules, the former results in approximately a 3% coupling, whereas the latter is about 26%.

LDL surface reactivity has been studied by a number of investigators, most of whom have used monofunctional reagents, and it is known that all the lysine residues will react under mild conditions [17]. The lack of reactivity in this study probably reflects a low physical binding of methotrexate by LDL, compared with its high binding by bovine serum albumin, which may facilitate the coupling reaction. This is supported by the fact that LDL has only been reported to bind lipid-soluble drugs [12].

The major physical effect of the coupling reaction is an increase in the measured radius and polydispersity of the LDL particles. The increase in radius could be ascribed to (a) the production of covalently linked aggregates or (b) actual size increases in the individual LDL particles. The former explanation is supported by the concomitant increase in polydispersity and the fact that LDL has been shown to have the ability to undergo a reversible aggregation [18] which, under the conditions of the experiments described here, could be made permanent by the crosslinking action of the carbodiimide. This probably also accounts for the losses during the coupling raction. It is also interesting that the larger the size increase the larger the increase in the NVD (polydispersity), a feature which supports explanation (a). The second explanation is unlikely, in view of previous work [26] in which the interaction of LDL with surfactants caused similar size increases without increases in the measured polydispersity, a result ascribed to increased particle asymmetry.

There appears to be no relationship between the quantity of methotrexate attached and the measured size increases or between the extent of aggregation occurring during the coupling reaction and the concentration of the reactants used. However, at the end of the reaction period the reactants are concentrated by ultrafiltration, and concentated LDL solutions have a greater tendency to aggregate [18].

The methotrexate LDL complexes, when tested against L1210 cells in vitro, are less active than free methotrexate, a feature which is common to most covalent macromolecular drug conjugates of methotrexate when tested under similar conditions [3]. It is likely that for the methotrexate to exert any effect it must first be released from the LDL complex either by hydrolysis in solution or by enzymatic hydrolysis once the LDL particle is phagocytosed and degraded. The latter pathway requires some form of receptor-dependent uptake, but we are unaware of any data on the LDL receptor capacity of L1210 and therefore unable to comment further. Hydrolysis in solution may also take place, especially during storage; but previous work has shown that this hydrolysis is likely to be negligible under the conditions of this study [11], although it may contribute to the overall inhibiting effect. However the reduction in activity demonstrated in Fig. 2, is comparable to that in other similar covalent systems and indicates that the methotrexate was attached to LDL, since the two curves differ.

The usefulness of LDL as a targeting vehicle is linked to its receptor-dependent uptake by tumour cells. Other experiments, however, have shown that attachment of materials to the exterior surface of LDL modifies receptor recognition [4], and this is obviously undesirable. A more promising approach would be to include the cytotoxic agent inside the particle, so leaving the surface properties intact. Nonetheless, this work has demonstrated the feasibility of using LDL as a carrier for cytotoxic drugs.

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