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Receptor dependent cellular uptake of synthetic low density lipoprotein by mammalian cells in serum-free tissue culture

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Abstract

Low density lipoprotein (LDL) is a normal plasma component, which is of interest in a number of research areas such as hypercholesterolaemia, drug targeting in cancer chemotherapy and as a lipid supplement in tissue culture systems. Currently, however, it can only be obtained by extraction from fresh plasma samples, which yields only small quantities. Synthetic LDL (sLDL) has been prepared using readily available lipid components coupled with a synthetic amphiphatic peptide molecule containing the apoprotein B receptor sequence. sLDL was capable of supporting the growth of Chinese Hamster Ovary (CHO) and fibroblast cells in serum-free culture media in a cholesterol-dependent manner that was related to the presence of the receptor peptide molecule. sLDL could be fluorescently labelled with 3,3′-dioctadecyloxalocarbocyanine perchlorate (DiO), and once labelled was assimilated by CHO and fibroblast cells in a time- and temperature-dependent manner that was dependent upon the presence of the receptor peptide. In addition, assimilation was reduced by an excess of unlabelled native LDL. The results indicated that the interaction of sLDL with CHO and fibroblast cells occurred via a receptor dependent system, most likely the LDL cellular receptor. sLDL is therefore a useful, easily obtained substitute for native LDL with potential applications in the areas of drug targeting to cells and serum-free tissue culture systems.

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Introduction

Low density lipoprotein (LDL) is a normal plasma component involved in the transport of cholesterol. It is composed of an internal core of cholesteryl esters, with small quantities of triglyceride, solubilized by a monolayer of phospholipids, which also contains small quantities of cholesterol and the apoprotein B (ApoB) receptor protein (Deckelbaum et al 1977). The receptor-dependent cellular interaction of LDL was elucidated by Brown & Goldstein (1986) during a series of seminal research papers on the biochemistry of familial hypercholesterolaemia. LDL is assimilated by cells through receptor dependent uptake, where the ApoB acts as the ligand and the cellular receptor is located in cell surface coated pits. After uptake the LDL is transported to the phagolysosomal system where it is digested and the cholesterol utilized for cellular metabolism. If sufficient intracellular cholesterol is accumulated this acts as a feedback mechanism inhibiting the de-novo intracellular synthesis of cholesterol and the further receptor dependent uptake of LDL.

The majority of LDL research is conducted in the field of hypercholesterolaemia and associated clinical conditions. However, research has also been undertaken into use as a drug targeting vector for cancer chemotherapy (Dubowchik & Firestone 1995) and as a lipid supplement for serum-free tissue culture systems (Blasey & Winzer 1989). In both of these areas research has been restricted by the requirement to obtain LDL from fresh plasma and the limited quantities available (Patsch et al 1974). The lipid constituents of LDL are readily available off the shelf and protein free analogues can be easily produced (Ginsburg et al 1982; Sjöström & Bergenståhl 1992; Owens & Halbert 1995). However, these systems lack ApoB and are not receptor competent.

The amphiphatic nature of ApoB provides an inherent resistance to classical peptide sequencing and its structure was only elucidated through cDNA analysis (Knott et al 1986;

Yang et al 1986). This identified a putative nine amino acid receptor site and placement of this hydrophilic sequence within an amphiphatic lipid construct produced a synthetic surface active ApoB receptor ligand (Owens et al 2001). The synthetic amphiphatic ApoB receptor construct interacts with protein-free LDL systems to produce peptide concentration- and chemical structure-dependent changes in particle size and zeta potential (Owens et al 2001). These synthetic LDL (sLDL) systems are also biologically competent and capable of supporting the proliferation and growth of U937 and NS0 cells in serum-free tissue culture systems (Baillie et al 2002; Hayavi & Halbert 2005). The U937 cell line possesses a biochemical defect and to proliferate has an obligate requirement for an extracellular source of cholesterol (Frostegård et al 1990). Normally in tissue culture this is provided via foetal bovine serum (FBS) supplementation or by the addition of native LDL, and U937 proliferation can be utilized as a measure of LDL receptor competency (Vandenbroek et al 1994). The ability of sLDL to permit proliferation in serum free media is indicative of sLDL assimilation by U937 via the LDL receptor (Baillie et al 2002). However, both U937 and NS0 grow as suspension cultures and this makes performance of classical LDL receptor binding experiments challenging (Hayavi & Halbert 2005), since these employ sessile cell systems.

The amphiphatic receptor peptide does not contain tyrosine and so sLDL is not amenable to classical LDL iodine radio-labelling techniques (Goldstein & Brown 1974). Fluorescent labelling of LDL with 3,3′-dioctadecyloxalocarbocyanine perchlorate (DiO) has been shown to be a quick and convenient method which does not alter the affinity of the lipoprotein for the LDL receptor (Stephan & Yurachek 1993). The lipid portions of the dye insert into the phospholipid monolayer whilst the conjugated chromophore lies on the particle's surface. This method of labelling is therefore applicable in situations where iodine labelling cannot be employed.

We have investigated the ability of sLDL to stimulate the proliferation of adherent cells in serum-free tissue culture media. In addition, the receptor based interaction of fluorescently labelled sLDL with the cell lines has been studied. This permitted the conduct of traditional cell uptake studies to determine the properties of the receptor interaction between sLDL and cells in tissue culture. This research has expanded the data on the number of cell lines known to interact with sLDL and has validated the ability of sLDL to target materials to cells via the LDL receptor pathway.

Materials and Methods

Materials

Cholesteryl esters, triglycerides, DiO (3,3′-dioctadecyloxalocarbocyanine perchlorate), phosphatidyl choline, sodium oleate, and potassium chloride were purchased from Sigma Aldrich (Poole, Dorset, UK). Dichloromethane was obtained from VWR International, Eastleigh, UK. Infinity Cholesterol, Infinity Triglyceride liquid stable reagent kit and phospholipid B assay kit were obtained from Alpha Laboratories, Eastleigh, UK. Synthetic peptides (Table 1) were obtained from Thistle Research (Glasgow, UK) at > 95% purity and were used without further purification.

Table 1 Peptide chemical structures used in the preparation of sLDL

Peptide	N-terminal	Sequence	C-terminal
1	Retinoic acid	Leu-Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu	Cholesterol
2	Retinoic acid	Gly-Thr-Thr-Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu	-COOH

Foetal bovine serum, gentamicin, fungizone, glutamine, trypsin-EDTA, Hams F10 and Dulbecco's modified Eagle's media were obtained from Sigma Aldrich (Poole, Dorset, UK). Chinese Hamster Ovary (CHO) protein-free medium and PC-1 fibroblast medium were obtained from Cambrex Bio Science Wokingham Ltd. (Wokingham, UK).

Cell lines

Chinese Hamster Ovary (CHO) cells (ECACC No. 85051005) and fibroblast (HFFF2) (ECACC No. 86031405) cells were acquired from European Collection of Cell Cultures (ECACC) as growing cultures. No subsequent modifications were made to the cells lines.

Preparation of sLDL

sLDL was prepared using a modified solvent evaporation technique previously reported by Hayavi & Halbert (2005). A 3:2:1 molar ratio of phosphatidylcholine, triglyceride and cholesteryl ester was dissolved in dichloromethane along with cholesterol and the synthetic peptide (Table 1) (at varying molar concentrations per mole of cholesteryl ester) added. To fluorescently label sLDL, DiO was added to the dichloromethane lipid mixture. The dichloromethane was then added to an aqueous solution of sodium oleate and homogenized using an ice-cooled EmusiFlex-C5 microfluidiser (Avestin, Canada) at pressures up to 30 000 psi. The organic solvent was then removed by evaporation at room temperature. The organic to aqueous phase ratio was kept at 1:9. The resulting system was filtered (0.2 μm) and stored aseptically at 4°C in the dark under a N_2 atmosphere.

The sLDL prepared as detailed above utilized the following ratios of the corresponding cholesteryl ester and triglyceride, oleic (21:41)/linoleic (50:15)/palmitic (12:25)/arachidonic (6:1.3)/stearic (0:5.7) with peptide expressed as the moles of peptide per mole of total cholesteryl ester.

Fluorescent labelling of LDL

LDL was labelled with DiO by a method modified from Stephan & Yurachek (1993). DiO stock solution (3 mg/5 mL in DMSO) was added to LDL solution at a concentration of

300 μg DiO (mg LDL protein^{-1}) and incubated in the dark at 37°C for five hours. After incubation the mixture was passed down a Sephadex G25M column (bed volume 9.1 mL) eluted with phosphate-buffered saline (PBS). Fractions (1 mL) were collected and analysed for fluorescence, excitation and emission wavelengths 484 nm and 587 nm, respectively, for DiO and also for total cholesterol content. For comparison, a similarly prepared DiO-labelled sLDL was subjected to chromatography.

Lipid measurements

The concentrations of total cholesterol (free + esterified), triglyceride and phospholipid were measured using the Infinity cholesterol reagent, Infinity triglyceride reagent and phospholipid B assay kit. The manufacturer's instructions were followed for each analysis.

Size and zeta potential measurements

sLDL was analysed by photon correlation spectroscopy using the ZetaSizer Model 4 (Malvern Instruments, Malvern, UK). Samples were diluted in distilled water and measurements carried out at a fixed angle of 90°. Results are expressed as the number mean ± s.d. (n = 10).

Cell culture

CHO stock culture was grown in Ham's F12 medium supplemented with 10% v/v FBS, glutamine (2 mM), fungizone (50 μg mL^{-1}) and pen-strep (0.1 μg mL^{-1}). Cells were seeded at 1–2 × 10^4 cells cm^{-2} using 0.25% v/v trypsin-EDTA, maintained in a humidified 5% v/v CO_2/air atmosphere at 37°C and sub-cultured twice weekly.

HFFF2 stock culture was grown in Dulbecco's modified Eagle's medium supplemented with 10% v/v FBS, glutamine 2 mM, fungizone (50 μg mL^{-1}) and pen-strep (0.1 μg mL^{-1}). Cells were seeded at 2–3 × 10^4 cells cm^{-2} using 0.25% v/v trypsin-EDTA, maintained in a humidified 5% v/v CO_2/air atmosphere at 37°C and sub-cultured twice weekly.

Cell growth assay

Assays were conducted in 96-well plates incubated at 37°C in a humidified 5% v/v CO_2/air atmosphere. One day before the experiment maintenance medium was replaced with serum- and lipid-free medium and cells incubated as detailed previously. For CHO cells, CHO protein-free animal component-free medium was employed and for fibroblasts PC-1 serum-free medium. Serum starved cells were harvested and seeded into wells at 1–2 × 10^4 cells cm^{-2} in the appropriate medium. After 72-h incubation the medium was replaced by MTT reagent (10 μL dissolved in 3 mL culture medium) and the plates incubated for a further 2–4 h. The resulting formazan crystals were then dissolved by adding 100 μL MTT solubilization solution and the absorbance measured spectrophotometrically at 570 nm using a Multiskan Ascent (Thermo Labsystems, Helsinki, Finland) plate reader. Background absorbance was measured at 690 nm and this was subtracted from the 570 nm measurement.

Statistical analysis

Cell growth experiment data were examined using Graph Pad Prism 4 for Macintosh, GraphPad Software Inc. (San Diego, CA). The results in Figure 1 were analysed using a two-way analysis of variance with a post analysis Bonferroni's test. The results in Figure 2 were analysed by a one-way analysis of variance with a post analysis Bonferroni's test.

Confocal microscopy

Before an experiment, cells were plated at 2 × 10^4 cells cm^{-2} in an eight-well chamber slide, 0.7 cm^2, 0.2 mL per chamber and then incubated as detailed above for cell growth assays. One day before the experiment maintenance medium was replaced with serum- and lipid-free medium. On the day of the experiment cells for 4°C incubation were chilled for 15 min before any additions. The wells were washed with PBS and the sLDL system in serum-free medium added. The slides were incubated for 1 or 3 h at 4 or 37°C, the latter in a humidified 5% v/v CO_2/air atmosphere. After incubation the cells were washed twice with ice-cold PBS containing bovine serum albumin (2 mg mL^{-1}), then fixed with 0.02 mL 2% w/v glutaraldehyde in PBS for 15 min at 4°C. The cells were

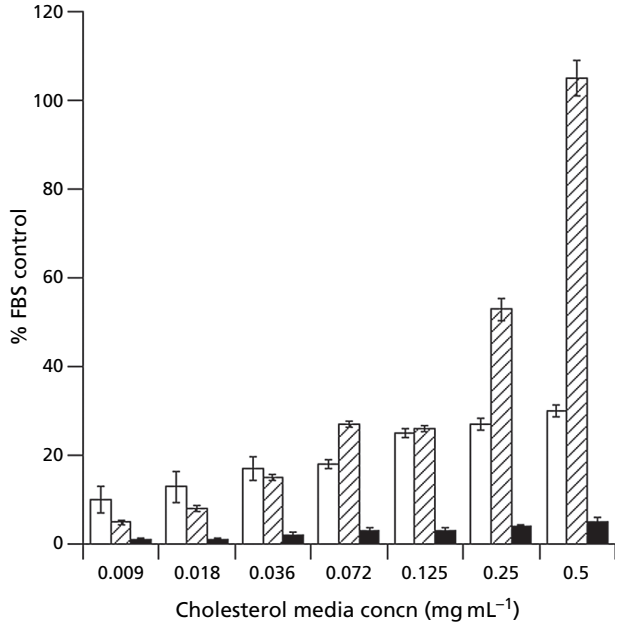

Figure 1 Proliferation of CHO cells induced by sLDL containing media after 72-h incubation. sLDL systems contained peptide **1** at varying concentrations, expressed as mol peptide per mol sLDL cholesterol, open bar 0.03, hatched bar 0.01, closed bar no peptide present. sLDL lipid constituents 3:2:1 molar ratio of phosphatidyl choline:triolein:cholesteryl oleate. Results (mean ± s.d., n = 8 wells) expressed as a percentage of a control medium system containing 10% v/v FBS supplementation. Peptide containing systems significantly different ($P < 0.001$) from peptide-free systems at all cholesterol concentrations tested. Peptide containing system at 0.03 mol peptide per mol sLDL cholesterol significantly different ($P < 0.001$) from system at 0.01 mol peptide per mol sLDL cholesterol except at cholesterol media concentrations of 0.036 and 0.125 mg mL^{-1} ($P > 0.05$).

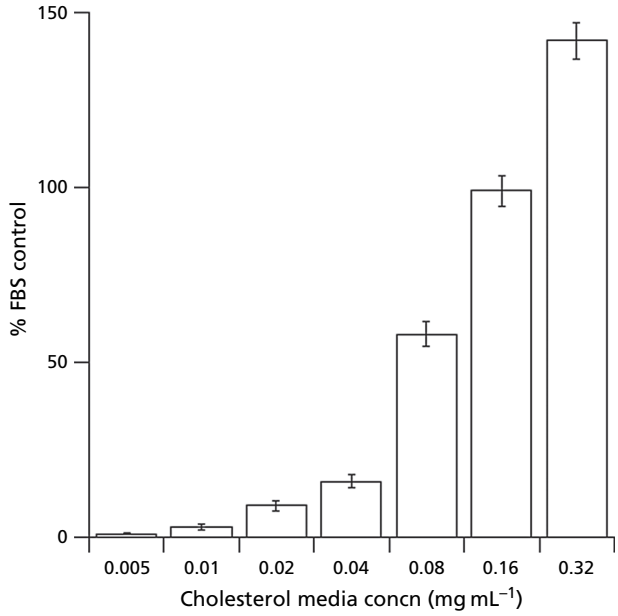

Figure 2 Proliferation of HFFF-2 fibroblast cells induced by sLDL containing media after 72-h incubation. sLDL system contained peptide **2** at a concentration of 0.03 mol peptide per mol sLDL cholesterol. sLDL lipid constituents 3:2:1 molar ratio of phosphatidyl choline:triolein:cholesteryl oleate. Results (mean ± s.d., n = 8 wells) expressed as a percentage of a control medium system containing 10% v/v FBS supplementation. Statistically significant difference ($P < 0.001$) between ascending cholesterol concentrations except between 0.005 and 0.01 ($P > 0.05$), 0.01 and 0.02 ($P < 0.01$).

finally washed twice with PBS and then visualized in a confocal microscope (Bio-Rad 1024, Bio-Rad) using a krypton–argon laser at an excitation wavelength of 488 nm with emission collected using a 525 nm (± 25 nm) filter. The lens employed was ×20 PA with a numerical aperture of 0.75 and the data analysed using "Laser Sharp" software (Bio-Rad).

Results and Discussion

sLDL-induced cellular proliferation

The ability of sLDL to support the proliferation of CHO and fibroblast cells in serum-free tissue culture medium is presented in Figures 1 and 2, respectively. When grown in media supplemented with 10% v/v FBS the cell numbers increased from the seeded value (10^4 cells cm^{-2}) to between 5×10^5–10^6 cells cm^{-2}, which was employed as the 100% control value. These results demonstrated that in serum-free media sLDL increased cellular proliferation in a cholesterol-dependent manner and in addition for CHO cells this was linked to the receptor peptide concentration present (Figure 1). The cholesterol-dependent nature of this effect was similar in character to those already demonstrated for the planktonic cell lines U937 (Baillie et al 2002) and NS0 (Hayavi & Halbert 2005). In addition, the CHO cell results indicated that the presence of the receptor peptide controlled the sLDL cellular interaction, since a peptide-free system was not as effective. These results were similar to results obtained for U937 and NS0 cells as was the proliferation of fibroblasts with a modified peptide lacking a C-terminal lipophilic substituent. However, there were some interesting differences with previous studies related to the magnitude of induced proliferation and the receptor peptide concentration. For example NS0 cell proliferation did not exhibit a maximal peptide concentration, whilst for CHO this was apparent at a receptor peptide concentration of 0.1 mol mol^{-1} cholesterol ester. Overall with CHO and fibroblasts the level of sLDL-induced proliferation was smaller than previously reported for NS0 cells (Hayavi & Halbert 2005). This was probably due to the biochemical differences between the two sets of cells. U937 and NS0 cells have an obligate requirement for an extracellular source of cholesterol to proliferate (Frostegård et al 1990) and therefore cannot proliferate in lipid-free media. The addition of lipid to U937 and NS0 cells therefore has a dramatic effect whilst for CHO and fibroblast cells, which are not dependent upon an extracellular source of cholesterol, the effect is not as dramatic. However, at the highest cholesterol concentrations tested the sLDL-induced growth stimulation was equivalent to that achieved by 10% v/v FBS supplementation.

DiO labelling

The labelling of native LDL with DiO had been performed by Stephan & Yurachek (1993). To determine if sLDL would behave in a similar manner, samples of both native LDL and sLDL were labelled using a modified literature method. The results of labelling are presented in Table 2 and Figure 3. The chromatography indicated that both systems eluted at the same time and that the DiO co-eluted with the cholesterol, indicating that it was bound to either particle. The overall analytical data indicated that DiO binding to sLDL was improved over that of native LDL in terms of efficiency of cholesterol and DiO recovery. This may reflect the simpler nature of the protein component of sLDL coupled with the well known instability of the native material. The results indicated that DiO could be employed to label sLDL in a similar fashion to the native material. Further studies demonstrated that DiO incorporation into the lipid mixture during sLDL production provided a simpler route to labelled sLDL (results not shown), which behaved identically to material labelled using the literature method.

Table 2 Recovery of cholesterol and DiO from PD-10 chromatography of LDL and sLDL

	Starting concn	Total recovered		Percentage recovered	
		LDL	sLDL	LDL	sLDL
Cholesterol	2.5 μM	1.5 μM	2.4 μM	58	96
DiO	600 μg	13.2 μg	124.6 μg	2	21

sLDL for this experiment was produced by the method of Owens et al (2001). Values were from a single experiment and measurement.

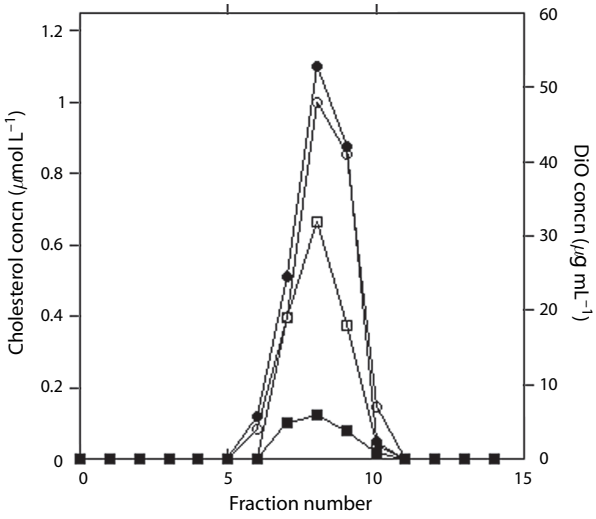

Figure 3 Chromatography of DiO labelled LDL and sLDL from a PD-10 gel exclusion column. Squares, LDL; circles, sLDL. Open symbols, cholesterol concentration; closed symbols, DiO concentration. Values were from a single experiment and measurement of each fraction.

Cellular uptake of DiO-labelled sLDL

Using fluorescently-labelled sLDL and fluorescent confocal microscopy it was easy to study the cellular interaction of the material (Figure 4). A control experiment using CHO cells incubated without the addition of any system indicated that the cells exhibited a small degree of autofluorescence (Figure 4A). The level of fluorescence was increased slightly if the cells were incubated with a fluorescently-labelled but receptor peptide-free sLDL system (Figure 4B). However, cells incubated with sLDL for 1 or 3 h at 37°C exhibited an obvious increased cell associated fluorescence over the previous control experiments (Figure 4C and D). This indicated that the presence of the peptide was important to the interaction. In addition, the intensity of cellular fluorescence increased during the incubation period. If a similar experiment was conducted at 4°C the level of cell associated fluorescence was reduced (Figure 4E) in comparison with the previous experiment at 37°C. The level of fluorescence after 3-h incubation at 4°C was lower than that achieved by the 1-h incubation at 37°C, indicating a much reduced cellular association of sLDL under these conditions. If the experiment was conducted in the presence of an excess of unlabelled native LDL the cellular fluorescence was again reduced (Figure 4F). A similar series of experiments could be conducted with fibroblast cells and the effect of temperature upon sLDL cell associated fluorescence is illustrated in Figure 4G and H. These results again illustrated that at 37 °C there was a substantial cell associated fluorescence which was markedly reduced by conducting the experiment at 4°C. In addition, cell morphology changed during the experiment with the greatest changes noted for cells incubated at 37°C with sLDL and an excess of native LDL. This was probably related to the uptake of sLDL and native LDL by these cells and the associated cellular biochemical sequelae occurring after uptake (see Introduction).

If sLDL cellular association occurs via the LDL receptor it should exhibit the classical properties of this system, that is

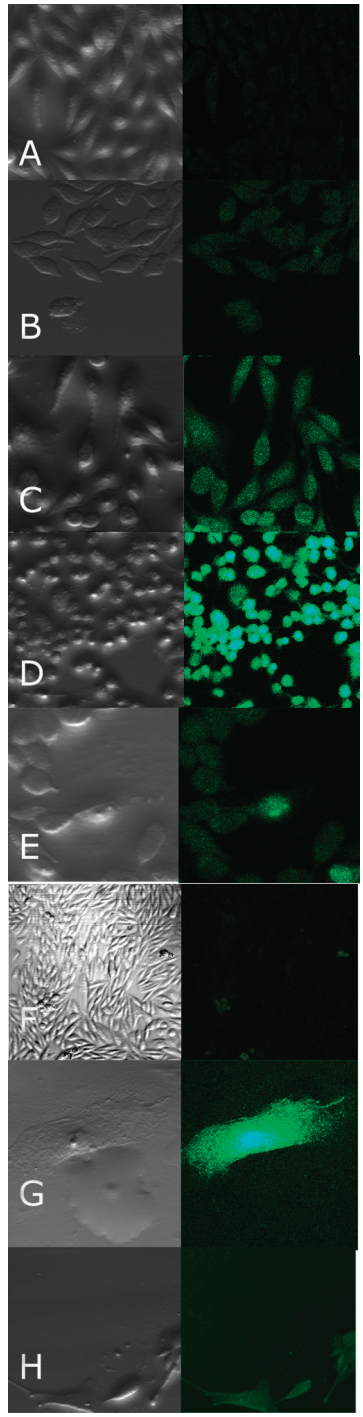

Figure 4 Confocal microscopy of DiO-labelled sLDL interaction with CHO and fibroblast cells. Left panels, picture normal microscopy; right panels, picture fluorescent microscopy. A: CHO control cells no treatment for 3 h. B: CHO cells with peptide free sLDL at 37°C for 3 h. C: CHO cells incubated with sLDL at 37°C for 1 h. D: CHO cells incubated with sLDL at 37°C for 3 h. E: CHO cells incubated with sLDL at 4°C for 3 h. F: CHO cells incubated with sLDL plus unlabelled native LDL 0.5 mg cholesterol mL^{-1} for 3 h. G: Fibroblasts incubated with sLDL at 37°C for 3 h. H: Fibroblasts incubated with sLDL at 4°C for 3 h. sLDL media concentration 0.036 mg cholesterol mL^{-1} containing 300 μg DiO mg^{-1} peptide.

time- and temperature-dependent uptake, which is subject to saturation or inhibition of uptake by large quantities of unlabelled material (Brown & Goldstein 1986). The results indicated that sLDL cell associated fluorescence was time dependent with a higher uptake at longer time intervals and also temperature dependent with a reduced uptake at low temperatures. The latter indicated that uptake was via an energy dependent pathway, indicative of uptake by the LDL receptor. Also the addition of an excess of unlabelled native LDL reduced sLDL cell associated fluorescence indicating a competitive interaction between sLDL and native LDL. This again indicated that sLDL cell associated fluorescence arose via an interaction with the LDL receptor. Previous results demonstrated that inclusion of an anti-LDL receptor antibody in media reduced the sLDL-induced proliferation of U937 and NS0 cells (Baillie et al 2002; Hayavi & Halbert 2005), again indicating interaction through the LDL receptor. Overall these results demonstrated that sLDL cell associated fluorescence was time and temperature dependent and reduced by competition with native LDL. Since a peptide-free sLDL system does not provide cell associated fluorescence, these results indicated that sLDL cellular interaction was via the LDL receptor.

Conclusions

The results illustrated that sLDL could act as a lipid source for CHO and fibroblast cells in serum-free tissue culture systems. The induced proliferation was related to the level of cholesterol (sLDL) added to the media and for CHO cells on the concentration of receptor peptide present on the sLDL. The maximal proliferation achieved was greater than that obtained by 10% v/v FBS supplementation indicating that sLDL could act as a substitute for FBS or a lipid supplement in serum-free media systems. Fluorescent studies indicated that sLDL could be labelled with DiO in a similar manner to native LDL; however, simple inclusion of the probe during manufacture would provide an easier route than current literature methods. The cellular interaction of DiO-labelled sLDL was time and temperature dependent, inhibited by excess native LDL and dependent upon the presence of the receptor peptide on the sLDL particle. These results and previous data indicated that sLDL interacted with cells via the LDL receptor. sLDL therefore has potential as a substitute for native LDL, as a drug targeting vector for cancer chemotherapy and as a lipid supplement for serum-free tissue culture systems. Further studies are required to fully elucidate the application of sLDL in these areas.

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