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EFFECTS OF MICROTUBULE-DISRUPTIVE AND MEMBRANE-STABILIZING AGENTS ON LOW DENSITY LIPOPROTEIN METABOLISM BY CULTURED HUMAN FIBROBLASTS

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Summary

The cellular mechanisms involved in the uptake and metabolism of low density lipoprotein (LDL) by cultured normal human fibroblasts have been investigated with the aid of drugs known to disrupt cytoplasmic microtubules or to inhibit membrane fusion.

Two drugs which disrupt microtubules by differing mechanisms, colchicine and vinblastine, each reduced the high affinity surface binding of ^{125}I-labelled LDL by fibroblasts. Associated reductions of the endocytosis and degradation of the lipoprotein could be attributed almost entirely to this effect. In contrast, lumicolchicine, an analogue of colchicine without microtubule-disruptive activity, had little or no effect on ^{125}I-labelled LDL metabolism.

Each of two groups of membrane-stabilizing agents, the phenothiazines and the tertiary amine local anaesthetics, directly inhibited both the internalization of ^{125}I-labelled LDL following high affinity binding to cell surface receptors and the catabolism of the lipoprotein subsequent to endocytosis, supporting previous morphological evidence for the importance of membrane fusion in these processes.

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Abbreviations: LDL, low density lipoprotein (density, 1.006—1.063 g/ml); HDL, high density lipoprotein (density, 1.063—1.21 g/ml).

Introduction

The surface membrane of normal human fibroblasts contains receptors for plasma low density lipoprotein (LDL) [1]. Binding of LDL leads to rapid internalisation of the lipoprotein and its incorporation into secondary lysosomes [2,3]. Here the protein component is degraded to trichloroacetic acid-soluble fragments, which are released from the cell [1], and the cholesteryl ester is hydrolysed [4]. The resultant free cholesterol inhibits cellular cholesterol synthesis [5] and the synthesis of LDL receptors, thereby reducing further LDL uptake [6]. The high efficiency with which LDL is internalized following binding appears to reflect the preferential localisation of the receptors in thickened and indented regions of the surface membrane (coated pits), specialised for rapid invagination [3].

We have recently shown that the internalisation of LDL receptors is inhibited by cytochalasin B, suggesting a role of cytoplasmic microfilaments in that process [7]. There is little other information, however, concerning the cellular mechanisms involved in LDL metabolism by fibroblasts. In view of the evidence for the participation of cytoplasmic microtubules in the control of cell surface topography [8—10] and in various cellular transport processes [11,12], we have investigated the effects of two microtubule-disruptive drugs, colchicine and vinblastine, on LDL metabolism by normal human fibroblasts, and have compared them with those of lumicolchicine, which does not disrupt microtubules but which possesses the other major biological activity of colchicine: inhibition of nucleoside transport [13]. In order to assess the importance of membrane fusion in LDL uptake and degradation, as suggested by the ultrastructural studies of Anderson et al. [3], the effects on ^{125}I-labelled LDL metabolism of two groups of membrane-stabilizing agents, the phenothiazines and the tertiary amine local anaesthetics [14], were also examined.

Methods

Materials. Eagle's basal medium [15], foetal calf serum and Dulbecco's phosphate-buffered saline [16] were obtained from Commonwealth Serum Laboratories, Melbourne. Carrier-free Na^{125}I, D-[U-^{14}C]sucrose (381 Ci/mol), sodium [1-^{14}C]acetate (61 Ci/mol) and [1α,2α-$^{3}H_2$]cholesterol (43 Ci/mmol) were purchased from Radiochemical Centre, Amersham, U.K. Polystyrene tissue culture ware was obtained from Falcon Plastics, Oxnard, CA, U.S.A. Porcine sodium heparin (grade II) and colchicine were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.; vinblastine sulphate from Eli Lilly and Co., Indianapolis, IN, U.S.A.; chlorpromazine from May and Baker Ltd., Dagenham, U.K.; and lignocaine from Astra Chemicals Ltd., Sydney, Australia. Instagel liquid scintillation counting solution was provided by Packard Ltd., Melbourne. Lumicolchicine was prepared by exposing a 0.25 mM solution of colchicine in 0.15 M NaCl to long-wave (366 nm) ultraviolet radiation for 36 h [17].

Cell cultures. Normal human skin fibroblasts at the 9th doubling number were obtained from the Commonwealth Serum Laboratories, Melbourne. Monolayers were maintained in 75 cm^2 flasks in a humidified incubator (95% air, 5% CO_2) at 37°C in Eagle's medium containing 10% (v/v) foetal calf serum (final concn: protein, 4 mg/ml; cholesterol, 0.9 mM), 10 mM $NaHCO_3$, 2 mM

glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin. Experiments were performed with cells grown to 70—90% confluency in 60 × 15 mm dishes, 4—6 days after seeding with $1—2 \cdot 10^5$ cells. At confluency there were approximately $1.5 \cdot 10^6$ cells/dish. Cells were studied between the 1st and 10th passages. Normal ^{125}I-labelled LDL metabolism by this cell line has already been characterised [7,18].

Lipoproteins and lipoprotein-deficient serum. Lipoprotein-deficient serum (density >1.21 g/ml; protein, 50 mg/ml; cholesterol, 35 nmol/ml) and LDL were prepared from fasting normolipidaemic human serum by preparative ultracentrifugation [19]. After dialysis against 20 mM Tris-HCl (pH 7.4)/0.15 M NaCl/0.3 mM EDTA and then against Dulbecco's phosphate-buffered saline, the solutions were sterilised by filtration (0.22 μm) and stored at 4°C.

Low density lipoprotein labelled with ^{125}I was prepared by a modification [20] of the IC1 method of McFarlane [21]. Specific activities of ^{125}I-labelled LDL were 156—456 cpm/ng protein. Less than 3% of the total ^{125}I in the final preparations was soluble in trichloroacetic acid or extractable into $CHCl_3$/CH_3OH (2 : 1, v/v).

Cellular uptake and degradation of ^{125}I-labelled LDL. At the end of an incubation with ^{125}I-labelled LDL, the medium was removed and the monolayers were rapidly cooled to 4°C. After precipitation of undegraded lipoprotein from the medium with trichloroacetic acid (final concn., 10%, v/v; 100°C, 5 min), an aliquot of the supernatant was treated to remove free iodide [22]. The residual radioactivity (after subtraction of the value obtained from an identical dish without cells) was used as a measure of the cellular release of the products of ^{125}I-labelled LDL proteolysis [19].

The monolayers were washed four times with 2 ml of 0.2% (w/v) bovine serum albumin/0.15 M NaCl and then twice with 4 ml of 0.15 M NaCl (both solutions at 0—4°C). Surface binding of ^{125}I-labelled LDL was quantified from the amount of radioactivity which was released from the cells during incubation (20 min, 4°C) with 2 ml of 0.6% (w/v) heparin in 0.15 M NaCl [2].

After release of surface-bound lipoprotein, the cells were dissolved in 2 ml of 0.1 M KOH (60 min, 37°C) and aliquots taken for measurement of residual total and non-iodide trichloroacetic acid-soluble radioactivity (representing, respectively, total and degraded intracellular ^{125}I-labelled LDL) and of cell protein [23], using albumin as standard. The extent of degradation was calculated as intracellular plus extracellular degraded lipoprotein. The sum of this and intracellular undegraded lipoprotein was considered to represent endocytosis. All data on ^{125}I-labelled LDL concentration, specific activity and metabolism are expressed in terms of protein content.

[^{14}C]Sucrose uptake. The uptake of sucrose was used as a measure of fluid endocytosis [24]. After incubation in medium containing 3.0 nmol/ml (1.14 μCi/ml) of D-[U-^{14}C]sucrose, monolayers were washed 10 times with 4 ml of 0.15 M NaCl (0—4°C) and dissolved in 2 ml of 0.1 M KOH (60 min, 37°C). Aliquots were then taken for protein determination [23] and for liquid scintillation counting in Instagel (counting efficiency, 80%). Clearance of [^{14}C]-sucrose (μl medium/mg cell protein) was calculated as: cellular uptake of radioactivity (cpm/mg) per concentration of radioactivity in the medium (cpm/μl).

Sterol and fatty acid synthesis. Following incubation of fibroblasts with or

without unlabelled LDL, sodium [1-^{14}C]acetate and unlabelled carrier acetate were added to the medium and the incubation continued for a further 1 h. The medium was then removed and the cells were washed 6 times with 2 ml of 0.15 M NaCl (0—4°C), and dissolved in 2 ml of 0.1 M KOH (60 min, 37°C). After removal of aliquots for protein assay [23], a known amount of [1α,2α-$^{3}H_2$]cholesterol was added. The mixture was saponified (1 M KOH in 70% ethanol for 3 h at 60°C), and the non-saponifiable lipids were extracted into 4 ml of hexane. The hexane was washed with 0.1 M sodium acetate and an aliquot taken for ^{14}C/^{3}H double-isotope counting (solvent: 15 ml toluene, 60 mg 2,5-diphenyloxazole, 0.75 mg 1,4-bis-2-(5-phenyloxazolyl)benzene; counting efficiencies: ^{14}C, 60%; ^{3}H, 37%). Sterol synthesis was quantified as the rate of incorporation of ^{14}C radioactivity into non-saponifiable lipids, after correction for the recovery of [^{3}H]cholesterol.

The aqueous phase of the saponification mixture was acidified with 1 ml of 12 M HCl. The fatty acids were then extracted into 4 ml of hexane, washed with 0.1 M sodium acetate, and assayed for ^{14}C radioactivity.

Results

The effects of adding increasing concentrations of colchicine to the culture medium on the metabolism of ^{125}I-labelled LDL by human fibroblasts are illustrated in Table I. Similar results were obtained in other experiments. The surface binding, endocytosis and degradation of lipoprotein were all inhibited at each drug concentration tested. The effects on binding and endocytosis were most marked (62—71% inhibition) at a colchicine concentration of 5 μM, above which a partial reversal of effect was observed. In contrast, the inhibition of ^{125}I-labelled LDL degradation showed no tendency to diminish as this concentration was exceeded.

Evidence that these effects of colchicine were a consequence of microtubule disruption was provided by other experiments (Table II). Vinblastine, which is

TABLE I

EFFECTS OF COLCHICINE ON THE METABOLISM OF ^{125}I-LABELLED LDL BY CULTURED HUMAN FIBROBLASTS

Cell monolayers were incubated for 18 h in medium containing 5% (v/v) lipoprotein-deficient serum. This was then replaced with fresh medium containing 5% lipoprotein-deficient serum, 10 μg/ml of ^{125}I-labelled LDL (specific activity, 170 cpm/ng) and the indicated concentration of colchicine. After further incubation for 3 h at 37°C the cells were harvested for measurement of surface binding, endocytosis and degradation of ^{125}I-labelled LDL as described under Methods. Results are expressed as the mean ± S.E. of five determinations or as the mean of duplicates. Mean cell protein/dish: controls, 285 μg; colchicine, 318 μg.

Colchicine concentration (μM)	ng ^{125}I-labelled LDL protein/mg cell protein		
	Surface binding	Endocytosis	Degradation
0	120 ± 5.2	3719 ± 174	1417 ± 52
1.25	64	1281	371
2.5	57	1270	402
5.0	45	1089	332
7.5	62	1346	335
10	75	1699	279

TABLE II

COMPARATIVE EFFECTS OF COLCHICINE, VINBLASTINE AND LUMICOLCHICINE ON ^{125}I-LABELLED LDL METABOLISM BY HUMAN FIBROBLASTS

Cell monolayers were incubated for 18 h in medium containing 5% (v/v) lipoprotein-deficient serum. This was then replaced with fresh medium containing 5% lipoprotein-deficient serum, 10 μg/ml of ^{125}I-labelled LDL (456 cpm/ng) and the indicated concentration of colchicine, vinblastine or lumicolchichine. After further incubation for 3 h at 37°C the cells were harvested for measurement of surface binding, endocytosis and degradation of ^{125}I-labelled LDL as described under Methods. Results are expressed as the means of duplicate determinations (coefficients of variation, 4—7%). Mean cell protein/dish: controls, 352 μg; colchicine, 345 μg; vinblastine, 326 μg; lumicolchicine, 359 μg.

Addition to medium	Concentration (μM)	ng ^{125}I-labelled LDL protein/mg cell protein		
		Surface binding	Endocytosis	Degradation
Nil	—	117	2714	2377
Colchicine	5.0	46	928	668
Vinblastine	12.5	61	1013	722
	50	55	437	284
	100	52	382	235
Lumicolchicine	5.0	100	2280	1984
	8.75	132	2466	2164
	12.5	100	2317	2024

known to disrupt microtubules by binding to a different site on the tubulin molecule from that to which colchicine binds [25], also inhibited the binding, endocytosis and degradation of ^{125}I-labelled LDL. In contrast, lumicolchicine, a structural analogue of colchicine which does not disrupt microtubules but which is a more potent inhibitor of nucleoside transport than is colchicine [13], had little or no effect on ^{125}I-labelled LDL metabolism.

The inhibition of LDL uptake by colchicine was associated, as would be anticipated, with a diminution of the suppression of sterol synthesis normally induced by LDL. Fibroblast monolayers, preincubated for 18 h in medium containing 5% (v/v) lipoprotein-deficient serum, were incubated for 5 h at 37°C in fresh medium containing 5% (v/v) lipoprotein-deficient serum plus: nil; LDL (25 μg protein/ml; 46 μg cholesterol/ml); or LDL plus colchicine (5 μM). Sodium [1-^{14}C]acetate and unlabelled carrier acetate were then added (2.5 μCi/ml; 0.1 μmol/ml). After a further 1 h at 37°C sterol radioactivity, measured as described under Methods, was significantly greater ($P < 0.01$) in cells incubated with LDL and colchicine (4558 ± 579 dpm/mg; mean ± S.E., $n = 4$) than in those incubated with LDL alone (2312 ± 173 dpm/mg), representing reductions of 62% and 81%, respectively, relative to control values (12 020 ± 282 dpm/mg). The possibility that this reflected a direct stimulation of sterol synthesis by the drug was excluded by other experiments in which colchicine alone (5 μM) reduced the incorporation of [^{14}C]acetate into sterols (but not into fatty acids) by approximately 40%, a finding consistent with a previous report that colchicine inhibited microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in rat liver cells [26].

The effects of the membrane-stabilizing agents, lignocaine and chlorpromazine, on ^{125}I-labelled LDL metabolism are illustrated in Table III. Similar results were obtained in three other experiments. Both drugs inhibited all mea-

TABLE III

EFFECTS OF LIGNOCAINE AND CHLORPROMAZINE ON ^{125}I-LABELLED LDL METABOLISM BY HUMAN FIBROBLASTS

Cell monolayers were incubated for 18 h in medium containing 5% (v/v) lipoprotein-deficient serum. This was then replaced with fresh medium containing 5% lipoprotein-deficient serum, 15 μg/ml of ^{125}I-labelled LDL (170 cpm/ng) and the indicated concentration of drug. After further incubation for 4 h at 37°C, the cells were harvested for measurement of surface binding, endocytosis and degradation of ^{125}I-labelled LDL as described under Methods. Results are expressed as the mean ± S.E. of six determinations or as the mean of duplicates. Mean cell protein/dish: controls, 284 μg; lignocaine, 285 μg; chlorpromazine, 265 μg.

Addition to medium (μM)	ng ^{125}I-labelled LDL protein/mg cell protein		
	Surface binding	Endocytosis	Degradation
Nil	260 ± 8	7234 ± 211	2559 ± 89
Lignocaine			
2	151	2150	99.4
3	118	1148	65.5
4	144	1265	60.9
7	139	965	54.1
Chlorpromazine			
20	172	3847	220
40	137	2178	244

sured parameters, lignocaine being the more potent, on a molar basis, by more than 10-fold. At each drug concentration tested the induced reductions of ^{125}I-labelled LDL binding (34—55%) were proportionately less than those of endocytosis (47—87%), which in turn were much less than those of degradation (90—98%). Similar changes were produced by procaine and promazine.

The nature of the reductions of ^{125}I-labelled LDL endocytosis induced by colchicine and chlorpromazine were further explored by examination of their effects on the cellular uptake of [^{14}C]sucrose, a measure of nonspecific pinocytosis [24]. In accordance with previous studies [27], colchicine had little or no effect on fluid endocytosis. Chlorpromazine, in contrast, was found to produce a substantial reduction of [^{14}C]sucrose clearance (Table IV). Nevertheless, estimates of the maximal contribution of nonspecific pinocytosis to ^{125}I-labelled LDL uptake, calculated as described previously [20], indicated that less than 5% of the drug-induced reduction of ^{125}I-labelled LDL uptake could be explained by the inhibition of this process.

The possibility, raised by the foregoing experiments, that membrane-stabilizing agents might directly inhibit both of the major stages of ^{125}I-labelled LDL metabolism subsequent to surface binding was supported by additional experiments, of which that illustrated in Table V is representative. When fibroblast monolayers were preincubated for 24 h in medium containing 5 μg/ml of ^{125}I-labelled LDL and were then washed and placed in fresh lipoprotein-deficient medium at 37°C, the subsequent reductions of surface-bound ^{125}I-labelled LDL and of intracellular undegraded ^{125}I-labelled LDL during 30 min of incubation were partially or completely prevented by the addition of chlorpromazine (40 μM) to the culture medium.

Finally, the mechanism by which colchicine reduces the surface binding of

TABLE IV

EFFECTS OF COLCHICINE AND OF CHLORPROMAZINE ON [^{14}C]SUCROSE CLEARANCE, ^{125}I-LABELLED LDL ENDOCYTOSIS AND THE MAXIMAL UPTAKE OF ^{125}I-LABELLED LDL BY PINOCYTOSIS IN CULTURED NORMAL HUMAN FIBROBLASTS

Fibroblast monolayers (cell protein, 260 μg) were preincubated for 18 h in medium containing 5% (v/v) lipoprotein-deficient serum. The medium was then replaced with fresh medium containing 5% lipoprotein-deficient serum and either 5 μg/ml of ^{125}I-labelled LDL (156 cpm/ng) or 3 nmol/ml of D-[U-^{14}C]-sucrose, with or without colchicine (5 μM) or chlorpromazine (20 μM). After further incubation for 20 h at 37°C, duplicate dishes were harvested for measurement of ^{125}I-labelled LDL binding and endocytosis or of [^{14}C]sucrose uptake, as described under Methods. Maximal ^{125}I-labelled LDL uptake as a consequence of pinocytosis was calculated as previously described [20].

Addition to medium	[^{14}C]Sucrose clearance (μl/mg)	Endocytosis of ^{125}I-labelled LDL (ng/mg)	
		By pinocytosis	Observed
Nil	4.7	102	3766
Colchicine	4.5	77	1425
Chlorpromazine	2.3	40	1563

^{125}I-labelled LDL by fibroblasts was further explored by examining the effect of the drug on the increase in ^{125}I-labelled LDL binding capacity which normally occurs during incubation of cells in lipoprotein-deficient medium [6]. As illustrated in Table VI, preincubation of cells for 24—72 h in medium containing lipoprotein-deficient serum without colchicine increased the binding of ^{125}I-labelled LDL during a subsequent 1 h incubation by 71—89%; when colchicine had been added to the preincubation medium (but not to the second incubation medium) the increment was only 15—29%.

TABLE V

EFFECTS OF CHLORPROMAZINE ON THE METABOLISM OF ^{125}I-LABELLED LDL ALREADY SURFACE BOUND OR INTERNALISED BY CULTURED HUMAN FIBROBLASTS

Fibroblast monolayers (mean cell protein, 314 μg) were preincubated for 24 h in medium containing 5% (v/v) lipoprotein-deficient serum and 5 μg/ml of ^{125}I-labelled LDL (456 cpm/ng protein). The dishes were then rapidly chilled to 4°C, the medium was removed, and the cells were washed twice with 4 ml of Dulbecco's phosphate-buffered saline (0—4°C). Some dishes were then harvested for measurement of surface bound and intracellular undegraded and degraded ^{125}I-labelled LDL as described under Methods. Other dishes were placed in fresh medium containing 5% lipoprotein-deficient serum with or without chlorpromazine (40 μM), incubated for a further 30 min at 37°C and then harvested. Results are expressed as the means ± S.E. of quadruplicate determinations. n.s., not significant.

	Incubation time (min)				
	0		30		
			No chlorpromazine		Chlorpromazine
Surface bound ^{125}I-labelled LDL (ng/mg)	98 ± 4.3	$P < 0.001$	41 ± 2.6	$P < 0.001$	77 ± 3.9
Intracellular undegraded ^{125}I-labelled LDL (ng/mg)	1660 ± 40	$P < 0.001$	1232 ± 30	$P < 0.001$	1610 ± 64
Intracellular degraded ^{125}I-labelled LDL (ng/mg)	70 ± 3.6	$P < 0.001$	37 ± 3.2	n.s.	46 ± 4.8

TABLE VI

EFFECT OF COLCHICINE ON THE INCREASE IN THE ^{125}I-LABELLED LDL BINDING CAPACITY OF FIBROBLASTS INDUCED BY PREINCUBATION OF THE CELLS IN LIPOPROTEIN-DEFICIENT MEDIUM

Fibroblast monolayers were grown to confluency in medium containing 10% (v/v) foetal calf serum and human LDL (10 μg protein/ml). Three dishes were then washed with phosphate-buffered saline, incubated for 1 h at 37°C in fresh medium containing 5% lipoprotein-deficient medium and 5 μg/ml of ^{125}I-labelled LDL (456 cpm/ng), and harvested for measurement of heparin-releasable radioactivity and cell protein as described under Methods. Other dishes were preincubated for 24, 48 or 72 h in medium containing 5% lipoprotein-deficient serum with or without colchicine (10 μM) before similar incubation in fresh medium containing ^{125}I-labelled LDL (in the absence of colchicine) and assay of heparin-releasable ^{125}I. Results are expressed as mean ± S.E. or as the mean of duplicates. Mean cell protein/dish: controls, 363 μg; colchicine, 349 μg.

Preincubation time (h)	Binding of ^{125}I-labelled LDL (ng/mg cell protein)	
	No colchicine	Colchicine
0	55 ± 5	—
24	94	63
48	98	68
72	104	71

Discussion

In these studies colchicine reduced the receptor-mediated surface binding, endocytosis and degradation of ^{125}I-labelled LDL by fibroblasts at concentrations known to disrupt cytoplasmic microtubules. Vinblastine, another microtubule-disruptive agent, had similar effects. In contrast, lumicolchicine, an analogue of colchicine which does not bind to tubulin but which does inhibit nucleoside transport [13], had little or no effect on ^{125}I-labelled LDL metabolism. Considered together these findings provide strong evidence that microtubules, or other tubulin-containing structures, participate in LDL metabolism by fibroblasts.

The percentage decrements induced by colchicine and vinblastine in the endocytosis and degradation of ^{125}I-labelled LDL were somewhat greater than those in surface binding. Nevertheless, examination of the data in Tables I and II indicates that the inhibition of binding was quantitatively the most important independent (i.e. direct) effect of the drugs. At a colchicine concentration of 5 μM, for example, the reduction of surface binding could account for 88—93% of the associated decrease in endocytosis of ^{125}I-labelled LDL, which in turn could account for 92% of the decrease in degradation *. Thus, tubulin appears to be involved principally in the receptor-mediated binding of LDL by fibroblasts, rather than in the mechanisms of internalization and degradation subsequent to binding, and to play a facilitative role in this process.

The precise nature of the involvement of tubulin in LDL binding cannot be determined from the present data. Previous studies have demonstrated that

* Calculations: fraction of total decrement in endocytosis attributable to reduction of surface binding, rather than to a direct effect on endocytotic mechanism = $\Delta B \cdot CE/\Delta E \cdot CB$; fraction of decrement in degradation attributable to reduction of endocytosis = $\Delta E \cdot CD/\Delta D \cdot CE$. Δ, decrement; C, control value; B, binding; E, endocytosis; D, degradation.

[^{3}H]colchicine binds to unidentified proteins in the plasma membrane of rat liver cells [28]. Thus, it is possible that tubulin shares structural similarities with the LDL receptor, and that colchicine and vinblastine reduce ^{125}I-labelled LDL binding by competing for the receptor. This possibility seems unlikely, however, for two reasons. Firstly, colchicine and vinblastine are known to bind to different sites on the tubulin molecule [29]. Secondly, the binding of ^{125}I-labelled LDL as a function of colchicine concentration did not appear to conform to a competitive phenomenon.

An alternative possibility is that microtubules participate in the transport of newly synthesized and recycled receptors through the cytoplasm to the plasma membrane. The probability that LDL receptors undergo extensive recycling following internalization has recently been discussed [30]. Inhibition of this process by disruption of microtubules would be expected to deplete the number of receptors on the cell surface, with a consequent reduction of ^{125}I-labelled LDL binding capacity. This possibility is supported by our additional finding that colchicine partially prevented the increase in the ^{125}I-labelled LDL binding capacity of fibroblasts which occurs during their prolonged incubation in lipoprotein-deficient medium. A function of microtubules in the cytoplasmic migration of LDL receptors would be analogous to their documented roles in the transport of catecholamine vesicles in the axons of neurones [31] and in the secretion of amylase by pancreatic acinar cells [12].

We have previously reported that cytochalasin B, a drug which selectively disrupts cytoplasmic microfilaments, produced reciprocal changes in the binding (increased) and endocytosis of ^{125}I-labelled LDL by cultured human fibroblasts, suggesting a function of microfilaments in facilitating the internalisation of receptor-LDL complexes [7]. Thus, microtubules and microfilaments may play reciprocal roles in the movement of LDL receptors between the surface membrane and the cytoplasm of fibroblasts.

This evidence for a role of microtubules in the control of LDL receptors extends the known function of these structures in lipoprotein metabolism. Other workers have already provided evidence for their participation in the secretion of very low density lipoprotein by the rat liver [32], in chylomicron secretion by the rat ileum [33], in the transport of lipoprotein lipase out of rat adipocytes [34] and in the uptake and catabolism of chylomicron remnants by the rat liver [35,36].

In contrast to the microtubule-disruptive drugs, which appeared to have little direct effect on either the endocytosis of ^{125}I-labelled LDL following surface binding or the subsequent catabolism of the lipoprotein, the phenothiazines and tertiary amine local anaesthetics inhibited both of these processes. The degree of inhibition of the internalization of receptor-LDL complexes was quantitatively similar to that of the clearance of [^{14}C]sucrose from the medium, a measure of pinocytosis. Phenothiazines and local anaesthetics are known to be potent inhibitors of membrane fusion [14]. Our observations thus provide pharmacological support for the conclusion drawn by Anderson et al. [3], on the basis of their ultrastructural studies with ferritin-labelled LDL, that the internalization of LDL receptors involves the fusion of adjacent areas of the plasma membrane, as the terminal event in the transformation of a coated pit into a coated endocytotic vesicle, and that the degradation of LDL following

endocytosis is mediated by the fusion of the vesicles with primary lysosomes.

The mechanism by which the phenothiazines and local anaesthetics reduced the surface binding of ^{125}I-labelled LDL is uncertain. One possibility is that it was related to the displacement of Ca^{2+} from the plasma membrane that appears to provide the basis of the membrane-stabilizing activity of these drugs [14], since divalent cations have been shown to be involved in the association of LDL with its receptor [30]. An alternative possibility, however, is that the finding was an artefact created by the 'trapping' of receptor-bound ^{125}I-labelled LDL in endocytotic 'flasks', resulting from the arrest of vesicle formation at the stage of fusion of the apposed surfaces of the plasma membrane. Such lipoprotein particles, although anatomically surface bound, might nevertheless be inaccessible to release by heparin. Ultrastructural studies would be required to confirm or refute this suggestion.

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