Degradation by cultured fibroblasts and macrophages of unmodified and 1,2-cyclohexanedione-modified low-density lipoprotein from normal and homozygous familial hypercholesterolaemic subjects

Brian L. KNIGHT and Anne K. SOUTAR

Medical Research Council Lipid Metabolism Unit, Hammersmith Hospital, DuCane Road,
London W12 0HS, U.K.

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Monolayer cultures of human skin fibroblasts and monocyte-derived macrophages were used to examine the effect of cyclohexane-1,2-dione modification on the proteolytic degradation of ^{125}I -labelled low-density lipoprotein (LDL) from normal subjects (NLDL) and homozygous familial hypercholesterolaemic subjects (FHLDL). Normal fibroblasts, pre-incubated in lipoprotein-deficient serum, and macrophages, pre-incubated in whole serum, exhibited both saturable and non-saturable degradation of LDL. In fibroblasts, the saturable receptor-mediated degradation of FHLDL was similar to that of NLDL and was abolished if the lipoproteins were modified with cyclohexanedione. The rate of non-saturable degradation of FHLDL was at least 3-fold higher than that of NLDL and each was decreased by approx. 60% after modification. In macrophages, saturable degradation was decreased but not abolished by modification. The apparent affinity for unmodified LDL was lower than that of the fibroblast receptor and was greater for NLDL than for FHLDL. Non-saturable degradation of FHLDL by macrophages was only slightly higher than that of NLDL. Modification with cyclohexanedione decreased the rate of non-saturable degradation of NLDL by 30%, but increased that of FHLDL by 75%. These experiments show differences between the degradation of ^{125}I -labelled NLDL and FHLDL. They suggest that macrophages can degrade LDL by a saturable process with different properties from that mediated by the fibroblast receptor and that, *in vitro*, the rate of degradation of the modified LDL is not the same as the rate of non-receptor-mediated degradation of unmodified LDL.

Cells cultured from a variety of human and animal tissues have the ability to synthesize surface receptors that bind extracellular LDL. Once bound, the LDL is rapidly transported into the cells and the protein and cholesteryl ester components are hydrolysed within lysosomes (Goldstein & Brown, 1977). Receptor-mediated binding and degradation of LDL can be prevented by blocking arginine groups of the apoprotein with cyclohexanedione (Mahley *et al.*, 1977). Cells from homozygous hypercholesterolaemic patients cannot synthesize significant numbers of active LDL receptors (Goldstein & Brown, 1977). However, these patients catabolize at least as much LDL per day as do normal subjects (Langer *et al.*,

1972). Thus other processes must play an important part in the degradation of LDL in the whole body. Non-receptor-mediated catabolism of LDL has been estimated *in vivo* from the rate of breakdown of LDL modified with cyclohexanedione with the assumption that the modification merely prevents receptor-mediated degradation and does not affect catabolism by other pathways (Shepherd *et al.*, 1979).

We have examined this assumption *in vitro* by using cultured human skin fibroblasts, which have been shown to possess LDL receptors, and macrophages, which might be expected to be particularly active sites of degradation that is not mediated by the LDL receptor. This paper describes the metabolism by these cells of cyclohexanedione-modified and unmodified LDL from both normal and homozygous hypercholesterolaemic subjects.

Abbreviations used: LDL, low-density lipoprotein (unspecified source); NLDL, low-density lipoprotein from normal subjects; FHLDL, low-density lipoprotein from homozygous familial hypercholesterolaemic subjects.

Methods and materials

Cells

Cell cultures were incubated at 37°C in an atmosphere of CO₂/air (1:19) in a humidified incubator. All culture medium was adjusted to pH 7.4 and contained 100 units of penicillin/ml and 100 µg of streptomycin/ml. Cells were routinely washed twice with Dulbecco's phosphate-buffered saline or Puck's saline A between changes in medium.

Fibroblasts derived from skin biopsies of normal and homozygous hypercholesterolaemic subjects were maintained in monolayer culture in Eagle's minimal essential medium with Hank's salts, 20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), 10 mM-NaHCO₃ and 10% (v/v) foetal-calf serum. For experiments, cells between the tenth and fifteenth passage were seeded at a concentration of 10⁵ cells/dish into 60 mm plastic Petri dishes containing 3 ml of Eagle's minimal essential medium with Earle's salts, 20 mM-N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), 25 mM-NaHCO₃ and 10% (v/v) foetal-calf serum. Cells were re-fed with fresh medium after 3 days. On the sixth day after seeding the cells were washed and incubated in 2 ml of medium containing lipoprotein-deficient serum (2.5 mg of protein/ml) in place of foetal-calf serum. Experiments were performed 24 h later when each dish contained approx. 300 µg of cell protein.

Human mononuclear cells were isolated from blood of normal donors by the methods described by Boyum (1968) and Johnson *et al.* (1977). Disodium EDTA was added to freshly drawn blood to give a final concentration of 1 mM. After centrifugation at 1000 g for 20 min the plasma was removed and an equivalent volume of phosphate-buffered saline (without Ca²⁺ and Mg²⁺) containing 1 mM-disodium EDTA was added. The reconstituted blood was layered on to Ficoll-Hypaque and centrifuged at 400 g for 45 min at 18°C. The band containing mononuclear cells was removed, diluted with 3 vol. of phosphate-buffered saline with 1 mM-disodium EDTA and centrifuged at 800 g for 10 min. The cells were resuspended and centrifuged at 100 g for 10 min. This washing procedure was repeated once using phosphate-buffered saline containing 1 mM-disodium EDTA and once with RPMI-1640 medium. The final pellet was resuspended in RPMI-1640 medium containing 10% (v/v) foetal-calf serum. About 6 × 10⁶ mononuclear cells were dispensed into 35 mm Multi-Dish wells containing a final volume of 2 ml of RPMI-1640 medium with 10% (v/v) foetal-calf serum. After 1 h at 37°C, non-adherent cells were removed by washing twice with RPMI-1640 medium. The adherent cells (approx. 20% of the cells added) were then incubated at 37°C in 1 ml of RPMI-1640 medium

containing 20% (v/v) autologous serum for up to 12 days with no further changes of medium. The few residual lymphocytes became detached after 24 h, when the monocytes were still rounded but firmly attached. Transformation into macrophages began between 3 and 5 days after seeding. The cells increased in size and assumed a variety of shapes with features characteristic of macrophage cultures (Johnson *et al.*, 1977). Experiments were performed with cells that had been cultured for 8–12 days, when over 90% of the monocytes had transformed into macrophages, but few giant cells were present. At this stage each well contained approx. 100 µg of cell protein.

Isolation and labelling of lipoproteins

Plasma from normal donors was obtained from freshly drawn blood containing 0.01% (w/v) disodium EDTA. The first 200 ml of plasma removed during plasma exchange (Thompson *et al.*, 1975) was used for the preparation of LDL from homozygous hypercholesterolaemic patients and, on one occasion, for the preparation of LDL from a normal subject. LDL (ρ 1.019–1.055 g/ml) was isolated from plasma by differential density centrifugation using methods described by Havel *et al.* (1955). The LDL was subjected to a second centrifugation at ρ 1.063 g/ml in the Beckman SW 50.1 rotor for 16 h at 43 000 rev./min, which resulted in a final background density for the isolated LDL of 1.055 g/ml. The LDL was dialysed for 3 h against at least four changes of buffer (0.15 M-NaCl/1 mM-disodium EDTA/0.01% NaN₃, pH 7.4) to remove the bulk of the KBr.

The dialysed LDL was immediately labelled with Na¹²⁵I using the method of McFarlane (1958) essentially as described by Bilheimer *et al.* (1975). The newly labelled LDL was separated from the reaction products by chromatography on Sephadex G-25 equilibrated with 0.15 M-NaCl/1 mM-disodium EDTA, pH 7.4, containing 0.01% (w/v) NaN₃, and then dialysed overnight against the same buffer without azide to remove residual non-bound iodide. Labelled LDL was then mixed with unlabelled LDL to give a specific activity of approx. 50 c.p.m./ng of protein, passed through a Millipore filter (0.45 µm pore diam.) and stored at 4°C. Freshly labelled ¹²⁵I-labelled LDL was treated with cyclohexane-1,2-dione under sterile conditions as described by Mahley *et al.* (1977). LDL (1 vol.; 5–10 mg of protein/ml) was mixed with 0.5 vol. of 0.3 M-cyclohexane-1,2-dione in 0.4 M-borate buffer, pH 8.1, and incubated for 2 h at 35°C. Excess cyclohexane-1,2-dione was removed by chromatography on Sephadex G-25 equilibrated with 0.15 M-NaCl/1 mM-disodium EDTA, pH 7.4, followed by dialysis overnight against the same buffer. Modified LDL was passed through a Millipore filter (0.45 µm),

stored at 4°C and used within 24 h. On some occasions ^{125}I -labelled LDL was incubated under the same conditions in 0.4 M-borate buffer without cyclohexanedione. Before each experiment ^{125}I -labelled LDL and modified ^{125}I -labelled LDL were dialysed for 2 h against phosphate-buffered saline. The specific activity of the labelled lipoproteins was then determined by the method of Lowry *et al.* (1951) to assay the protein concentration, with bovine serum albumin as standard.

Lipoprotein-deficient serum

Lipoprotein-deficient serum was prepared from the infranatant solution obtained after isolation of LDL from normal donors. The density of the infranatant was adjusted to ρ 1.21 g/ml by the addition of solid KBr and lipoproteins of ρ < 1.21 g/ml were removed by tube-slicing after centrifugation for 40 h at 55 000 rev./min in a Beckman 60 Ti rotor. KBr was removed by extensive dialysis against phosphate-buffered saline after which the lipoprotein-deficient plasma was treated with thrombin (20 units/ml) for 15 min at room temperature. The clot was removed by centrifugation and filtration through Whatman no. 1 paper. The lipoprotein-deficient serum was sterilized by filtration through a Millipore filter (0.22 μm) and stored frozen at -20°C.

Experimental procedure

Immediately before the experiments, medium was replaced with fresh medium containing lipoprotein-deficient serum (2.5 mg of protein/ml). Lipoproteins were added to give a final volume of 2 ml in 60 mm dishes or 1 ml in 35 mm Multi-Dish wells, and the incubations were continued for up to 5 h at 37°C. At the end of the incubations the medium was removed. Macrophages were washed twice with 0.15 M-NaCl/50 mM-Tris/HCl (pH 7.4) and dissolved in 0.1 M-NaOH for the assay of protein. Fibroblasts were extensively washed as described by Goldstein *et al.* (1976). For assay of LDL binding the washed cells were incubated with heparin as described by Goldstein *et al.* (1976). After removal of the heparin solution, which contained the surface-bound radioactivity, cells were washed and dissolved in 0.1 M-NaOH for assay of internalized LDL and protein. For assay of total cell-associated radioactivity, washed cells were dissolved in 0.1 M-NaOH without heparin treatment. Acid-soluble, non-iodide products of lipoprotein catabolism were assayed in samples of medium after precipitation with 10% (w/v) trichloroacetic acid and two extractions with chloroform (Goldstein & Brown, 1974). Values were corrected for breakdown that occurred in medium alone to give the amount of lipoprotein degraded by the cells. The rate of degradation exhibited by macrophages was constant throughout the 5 h incubation. The rate exhibited by

fibroblasts was constant after a lag of approx. 1 h. Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Cell-free extracts were prepared as described by Goldstein *et al.* (1975). Degradation of lipoprotein was assayed for 1 h at 37°C in a total volume of 100 μl containing 0.1 M-sodium acetate buffer (pH 4.0)/5 mM-dithiothreitol/1 mM-disodium EDTA and approx. 40 μg of extract protein. The reaction was stopped and the degradation products were determined as described by Goldstein *et al.* (1975).

Materials

All constituents of tissue culture medium were obtained from Gibco Europe, Paisley, Scotland, U.K. Linbro Multi-Dishes (6 $\times$ 35 mm) were from Flow Laboratories, Irvine, Scotland, U.K., and 60 mm $\times$ 15 mm Contour Petri dishes from Lux Scientific Corp., 1000 Oaks, CA, U.S.A. Ficoll-Hypaque was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and Na^{125}I (carrier-free) from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Degradation by cultured skin fibroblasts

Monolayers of cultured human fibroblasts were incubated for 24 h in medium containing lipoprotein-deficient serum and then for 5 h in the presence of different concentrations of ^{125}I -labelled NLDL or FHLDL. Degradation of NLDL and FHLDL by normal fibroblasts (Fig. 1b) increased rapidly as their concentration in the medium was raised. The rate of increase then slowed and, at higher concentrations, remained constant. We have interpreted the shape of these curves to indicate that the degradation of LDL can be divided into two categories, each defined by its response to increasing concentrations of LDL. The linear part of the curve represented non-saturable degradation that occurred throughout the concentration range at a rate (ng/5 h) that was directly proportional to the concentration of LDL. Superimposed upon this was degradation mediated by a mechanism that was essentially saturated by 50 μg of LDL protein/ml. This saturable mechanism was responsible for the high rate of degradation of low concentrations of LDL by normal fibroblasts and had little or no activity in fibroblasts from homozygous hypercholesterolaemic patients (Fig. 1d).

The rate of non-saturable degradation of LDL was estimated from the slope of the linear part of concentration curves such as those shown in Fig. 1. Results from a number of independent experiments are given in Table 1. In normal fibroblasts, non-saturable degradation of FHLDL was much greater than that of NLDL. This difference was observed with FHLDL from four patients. In each case the

Fig. 1. Relationship between LDL concentration and the amount of LDL associated with (a and c) and degraded by (b and d) skin fibroblasts derived from normal (a and b) and homozygous hypercholesterolaemic (FH; c and d) subjects. Cells were preincubated for 24 h with lipoprotein-deficient serum and for 5 h at 37°C with different concentrations of 125 I-labelled NLDL (O), cyclohexanedione-modified NLDL (●), FHLDL (Δ) or cyclohexanedione-modified FHLDL ($\blacktriangle$). The specific activity of each LDL was approx. 40 c.p.m./ng of protein. Cell-associated radioactivity is the sum of that bound and internalized and has not been corrected for non-specific association. Values given are means of triplicate incubations.

Table 1. Rates of non-saturable degradation of unmodified and cyclohexane-1,2-dione-modified LDL by cultured fibroblasts and monocyte-macrophages

Fibroblasts were pre-incubated for 24 h in medium containing lipoprotein-deficient serum (2.5 mg of protein/ml). Human macrophages were cultured for 8–12 days in medium containing 20% (v/v) autologous serum. Values of non-saturable degradation were calculated from the slope of the linear parts of concentration curves such as those shown in Fig. 1 and Fig. 3, and are given as means $\pm$ S.E.M., with the number of pairs given in parentheses after the corresponding ratio. The ratio is given as the mean $\pm$ S.E.M. of the ratios within each experiment. Results shown are derived from separate experiments, each employing cultured fibroblasts or macrophages from one of three normal donors or two Type IIa (receptor-deficient; Goldstein & Brown, 1977) familial hypercholesterolaemic patients. Each fibroblast experiment employed a preparation of LDL from a different donor. Macrophage experiments used LDL from one of three normal or three familial hypercholesterolaemic subjects. * Significant effect of modification ($P < 0.05$, paired t test). † FHLDL significantly greater than NLDL ($P < 0.01$, Student's t test).

Cell type	Non-saturable degradation (ng/mg of protein per 50 μ g increase in LDL concn. per ml) of:					
	NLDL			FHLDL		
	Unmodified	Modified	Modified/unmodified	Unmodified	Modified	Modified/unmodified
Normal fibroblasts	79 $\pm$ 23	33 $\pm$ 11*	0.40 $\pm$ 0.05 (4)	311 $\pm$ 67†	132 $\pm$ 27*	0.43 $\pm$ 0.05 (4)
Familial hypercholesterolaemic fibroblasts	123 $\pm$ 2	46 $\pm$ 12*	0.37 $\pm$ 0.09 (3)	344 $\pm$ 94	173 $\pm$ 32	0.53 $\pm$ 0.09 (3)
Human macrophages	128 $\pm$ 36	85 $\pm$ 24*	0.66 $\pm$ 0.04 (7)	192 $\pm$ 39	338 $\pm$ 72*	1.74 $\pm$ 0.22 (5)

rate was at least three-fold higher than that of NLDL determined at the same time. Non-saturable degradation of NLDL was greater in fibroblasts from homozygous hypercholesterolaemic patients

than in normal fibroblasts, but was still less than that of FHLDL. Modification of the apoprotein with cyclohexanedione not only prevented the saturable degradation of both FHLDL and NLDL, but also

decreased the rate of non-saturable degradation of each lipoprotein by over 50% (Table 1). This effect of modification on the rate of non-saturable degradation was observed with both normal and mutant cells and could not be explained by changes unrelated to the coupling of cyclohexanedione to the apoprotein. Incubation without cyclohexanedione under the conditions used for modification had no effect on the subsequent rate of degradation of either FHLDL or NLDL.

The rate of saturable degradation of LDL was calculated as the difference between the observed rate at a given concentration and the non-saturable rate at the same concentration, estimated from the linear part of the concentration curve. Fig. 2 shows the saturable degradation and receptor-mediated binding and internalization of FHLDL and NLDL by normal fibroblasts. Saturable degradation of FHLDL was the same as that of NLDL, with one-half maximal activity at approx. $10\mu\text{g}$ of LDL protein/ml. There was also no differences between FHLDL and NLDL in the amount of lipoprotein bound and internalized by the cells. The data confirm that the lack of saturable degradation of the cyclohexanedione-modified lipoproteins results from their inability to bind to the receptors.

The relationship between the concentration of LDL and the amount that was associated with the fibroblasts at the end of the incubations is shown in Fig. 1(a) and Fig. 1(c). Approx. 90% of the radioactivity represented LDL that had been internalized by the cells. Saturable degradation of FHLDL and NLDL was accompanied by a similar concentration-dependent accumulation of LDL by the cells. However, there were no differences in cell-association between FHLDL and NLDL that corresponded to the observed differences in their rates of non-saturable degradation. These results suggested that non-saturable degradation was not accompanied by a net accumulation of LDL within the cells. To check that it was not catalysed by proteolytic enzymes released from the fibroblasts, LDL was incubated with medium that had been in contact with cells for 24h. Degradation of LDL in the medium was less than 10% of that observed in the presence of cells.

Degradation by cell-free extracts

Cell-free extracts, prepared by sonication of harvested fibroblasts, degraded LDL at acid pH values. With up to 1mg of extract protein/ml the rate of formation of acid-soluble proteolytic products was constant for at least 90min, was directly proportional to the concentration of extract and increased linearly with increasing concentrations of LDL up to $250\mu\text{g}$ of LDL protein/ml. The rate of degradation of FHLDL was essentially the same as that of NLDL and was not affected by modification

of either LDL with cyclohexanedione. A similar result was obtained with extracts from human macrophages.

Degradation by human macrophages

Macrophages are assumed to be important sites for the degradation of LDL that is not mediated by

Fig. 2. Receptor-mediated surface binding (a), internalization (b) and degradation (c) of LDL by normal skin fibroblasts

Cells were pre-incubated for 24h with lipoprotein-deficient serum and for 5h at 37°C with different concentrations of cyclohexanedione-modified (closed symbols) or unmodified (open symbols) NLDL (circles) or FHLDL (triangles). The specific radioactivity of each LDL was approx. 30 c.p.m./ng of protein. Results obtained from incubations of up to $50\mu\text{g}$ of radioactive LDL/ml in the presence of an excess (1mg/ml) of the corresponding unlabelled LDL were used to correct for non-specific binding and internalization. Degradation was calculated as the difference between the observed values and those for non-saturable degradation at the same concentration derived from the slope of the linear part of the concentration curve (see Fig. 1). Values given are means of duplicate incubations.

the specific LDL receptor. We therefore studied the catabolism of LDL by human macrophages under conditions in which the synthesis of LDL receptors had not been deliberately induced. The relationship between the concentration and degradation of unmodified LDL by cultured macrophages grown in whole serum is shown in Fig. 3. Again the shape of the curve suggested that the degradation could be separated into two types. One type was saturated by approx. $300\mu\text{g}$ of LDL protein/ml, whereas the rate of the other type remained directly proportional to the amount of LDL up to a concentration of 1 mg/ml . The slope of the linear portion was used to estimate the rate of non-saturable degradation. The rate of saturable degradation could then be calculated as the difference between the total observed rate and the non-saturable rate at the same concentration. Experiments with medium that had been in contact with macrophages for at least 16 h suggested that none of the degradation could be attributed to enzymic activity released from the cells.

Total degradation of low concentrations of NLDL and FHLDL by macrophages is shown in Fig. 4. FHLDL was degraded more slowly than was NLDL, although the proportional difference diminished as the concentration increased. This difference between NLDL and FHLDL resulted from an apparent difference in the affinity for LDL from the two

sources. In four experiments, each employing a different preparation of LDL from one of three normal donors or four homozygous hypercholesterolaemic patients, the protein concentration that gave one-half of the maximal rate of saturable degradation was $28 \pm 4\mu\text{g/ml}$ for NLDL and $63 \pm 10\mu\text{g/ml}$ for FHLDL (mean $\pm$ s.e.m.). Saturable degradation of both types of ^{125}I -labelled LDL could be completely inhibited by an excess of either unlabelled NLDL or unlabelled FHLDL.

Total degradation of NLDL was decreased after modification with cyclohexanedione, but the shape of the concentration curve indicated that some saturable degradation remained (Fig. 4). The rate of degradation of modified FHLDL was greater than that of modified NLDL and was almost as high as that of unmodified FHLDL. However, it was apparently directly related to the concentration, suggesting that the rate of non-saturable degradation was particularly high. This was confirmed by studies with higher concentrations of LDL, such as that shown in Fig. 5. At these concentrations saturable degradation was maximal (Fig. 3) and the slope of the line represented the rate of non-saturable degradation. Results from a number of experiments

Fig. 3. *Relationship between the concentration of LDL and its rate of degradation by cultured macrophages* Human cells were cultured for 10 days in medium containing 20% (v/v) autologous serum. Immediately before the experiment the macrophages were washed and medium containing lipoprotein-deficient serum (2.5 mg of protein/ml) was added. The cells were then incubated for 5 h at 37°C with different concentrations of ^{125}I -labelled FHLDL (11 c.p.m./ng of protein). Values are means of duplicate incubations.

Fig. 4. *Degradation of low concentrations of low-density lipoproteins by cultured macrophages*

Human cells were cultured for 8 days in medium containing 20% (v/v) autologous serum. Immediately before the experiment the macrophages were washed and medium containing lipoprotein-deficient serum was added. Cells were then incubated for 5 h at 37°C with different concentrations of ^{125}I -labelled LDL (30 c.p.m./ng of protein for NLDL and 40 c.p.m./ng of protein for FHLDL). Values are shown for NLDL ($\circ$), cyclohexanedione-modified NLDL ($\bullet$), FHLDL ($\triangle$) and cyclohexanedione-modified FHLDL ($\blacktriangle$) and are means of duplicate incubations.

Fig. 5. Degradation of high concentrations of low-density lipoprotein by cultured macrophages

See the legend to Fig. 4 for details. Human cells had been cultured for 12 days before the experiment. The specific radioactivity of NLDL was 12 c.p.m./ng of protein and of FHLDL was 7 c.p.m./ng of protein. Values are shown for NLDL (○), cyclohexanedione-modified NLDL (●), FHLDL (△) and cyclohexanedione-modified FHLDL (▲) and are means of duplicate incubations.

are summarized in the bottom line of Table 1. In five experiments, each employing a separate preparation of FHLDL from one of three patients, the rate of non-saturable degradation of cyclohexanedione-modified FHLDL was always greater than that of unmodified FHLDL. In contrast, non-saturable degradation of modified NLDL was always less than that of unmodified NLDL. Non-saturable degradation of FHLDL was slightly, but not significantly, greater than that of NLDL.

Discussion

The characteristics of the cell-surface receptor that mediates the saturable degradation of LDL in fibroblasts are well documented (Goldstein & Brown, 1977). Our results confirm that the receptor does not distinguish between NLDL and FHLDL (Goldstein & Brown, 1973) and does not bind LDL that has been modified with cyclohexanedione

(Mahley *et al.*, 1977). Human monocyte-derived macrophages also exhibited saturable degradation of LDL, although its apparent affinity for LDL was less than that of the fibroblast receptor and was greater for NLDL than for FHLDL. Modification with cyclohexanedione decreased, but did not abolish, saturable degradation of NLDL by macrophages. Thus saturable degradation of LDL in macrophages did not occur through a mechanism identical with that in fibroblasts. Indeed, the cells were cultured in whole serum, which, in fibroblasts at least, represses the expression of LDL receptors (Goldstein & Brown, 1977). In two previous studies that have shown saturable degradation of LDL in human macrophages, the concentration required to give one-half of the maximal rate was 14 µg/ml in cells pre-incubated without serum (Traber & Kayden, 1980) and approx. 40 µg/ml in cells grown with 30% (v/v) autologous serum (Fogelman *et al.*, 1980). Our value for NLDL obtained with cells grown in 20% (v/v) serum was 28 µg/ml. It is possible that the properties of the mechanism responsible for saturable degradation can be influenced by the prevailing cellular environment which, in turn, depends on the conditions of incubation. There is, at present, no conclusive evidence to indicate whether the saturable mechanism in macrophages involves some component with the physical properties of the classical LDL receptor found in fibroblasts or whether it is a distinct process that could contribute to the LDL-receptor-independent degradation observed *in vivo*.

In fibroblasts, non-saturable degradation of FHLDL was greater than that of NLDL. Each was decreased by approx. 60% after modification with cyclohexanedione. The observed rate of non-saturable degradation could be the sum of a number of processes. Our experiments indicate that little degradation can be attributed to enzymes released from the cells although it is possible that some could be catalysed by enzymes on the outer surface. Moreover, the observed differences cannot be explained by differences in the rate of degradation once the lipoproteins have entered the cell. Simple ingestion of medium during pinocytosis would give the same rate of degradation of each variety of LDL. This cannot be greater than the lowest rate observed, that of modified NLDL, and was particularly low in fibroblasts from normal subjects. Selectivity and degradation additional to that mediated by fluid pinocytosis must occur through other processes. Miller *et al.* (1978) have proposed that fibroblasts can take up LDL by adsorptive endocytosis involving non-specific association of the lipoprotein with the cell surface. This could explain the differences in the rates of non-saturable degradation by fibroblasts. Adsorption could be greater for FHLDL than for NLDL and decreased by the

changes in charge or structure that result from modification with cyclohexanedione.

In macrophages the differences in non-saturable degradation between NLDL and FHLDL, and between NLDL and modified NLDL, were smaller than in fibroblasts, indicating that fluid pinocytosis may account for a greater proportion of the total rate of non-saturable degradation. Macrophages also differed from fibroblasts in their non-saturable degradation of modified FHLDL. Whereas modification with cyclohexanedione decreased the rate of degradation of NLDL, it increased the degradation of FHLDL. This is unlikely to have resulted from a difference in the extent of modification of the LDL from the two sources. The modification produced the same decrease in non-saturable degradation in fibroblasts, and neither modified lipoprotein bound to the fibroblast receptor. Presumably the modification procedure produced changes in the structure of the FHLDL that rendered it more accessible to some process in macrophages that is not present in fibroblasts. Human macrophages possess a variety of surface receptors, including those that can bind LDL that has been acetylated or treated with malonaldehyde (Fogelman *et al.*, 1980). It is possible that modified FHLDL, but not modified NLDL, was taken up with low affinity through one of these receptors.

It is clear from these results that at least part of the FHLDL differs in some way from NLDL. However, there is not, at present, any obvious explanation for the observed differences in catabolism of the two varieties of LDL. The lipid composition of FHLDL is known to differ from that of NLDL, probably as a result of its prolonged half-life in the circulation of homozygous hypercholesterolaemic patients (Jadhav & Thompson, 1979). FHLDL contains relatively more cholesterol, especially non-esterified cholesterol, than does NLDL and its ratio of sphingomyelin to phosphatidylcholine is higher. However, no differences have been detected in their apoB moieties, nor in their immunological properties. We cannot exclude the possibility that the differences in catabolism may not be exhibited by the native lipoproteins *in vivo* but result from differential effects produced by the procedures used to isolate and label the LDL. NLDL isolated from plasma obtained during plasma exchange of a normal subject gave the same results as NLDL isolated from freshly drawn blood. Thus the differences cannot be attributed to the use of the products of plasma exchange for the preparation of FHLDL.

It is difficult to extrapolate from results obtained with cyclohexanedione-modified LDL *in vitro* to studies conducted in the whole body. The sites of LDL-receptor-independent degradation of LDL in the body are not known and may not involve any of

the mechanisms that we have been studying here. Moreover, the modified LDL is injected into a vastly greater pool of native LDL which may influence the way in which it is catabolized. Nevertheless, our experiments show that non-saturable degradation of cyclohexanedione-modified LDL by both fibroblasts and macrophages is not the same as that of the corresponding unmodified LDL. Thus, although the catabolism of modified LDL may be qualitatively related to the degradation of native LDL through pathways that do not involve the LDL receptor, it should not be regarded as a reliable quantitative measurement of the activities of these pathways *in vivo*.

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