Low-density-lipoprotein receptors in human fibroblasts are not degraded in lysosomes

Livia A. F. CASCIOLA, Kevin I. GRANT, Wieland GEVERS, Gerhard A. COETZEE and Deneys R. VAN DER WESTHUYZEN*

MRC/UCT Research Unit for the Cell Biology of Atherosclerosis, Department of Medical Biochemistry, University of Cape Town Medical School, Observatory, Cape Town, 7925, South Africa

The rate of degradation of low-density-lipoprotein (LDL) receptors was measured in cultured human skin fibroblasts by [³⁵S]methionine pulse-chase experiments. The half-life of LDL receptors was unaltered by inclusion of LDL in the medium (*t*_{1/2} 11 h). Neither lysosomotropic inhibitors (chloroquine or NH₄Cl) nor leupeptin inhibited the rate of receptor degradation in the absence of ligand. In cells incubated at 18 °C to inhibit the delivery of internalized ligands from endocytic vesicles to lysosomes, receptor degradation continued, but at the expected rate of about six times lower than that at 37 °C. Mutant LDL receptors defective in internalization were degraded at the same rate as normal receptors, suggesting that receptor internalization and recycling are not required for basal turnover. We conclude that the rate-limiting steps for, and probably the whole pathway of, degradation of normal LDL receptors does not take place in lysosomes.

INTRODUCTION

Low-density-lipoprotein (LDL) receptors have been extensively studied, and much is known about their synthesis, processing and recycling (reviewed in [1]). As is the case for many other cell-surface receptors, however, the cellular site(s) and biochemical mechanism(s) responsible for degradation of these receptors have not been elucidated [2]. In many receptor systems, internalized ligands are delivered intact to lysosomes and broken down there, but this is not necessarily coupled to the degradation of receptors. Certain receptors, such as those for LDL, transferrin and the asialoglycoproteins, undergo repeated endocytic recycling (reviewed in [3]). These receptors internalize their ligands and then recycle separately back to the plasma membrane from acidic endosomal compartments. Other receptors, typified by those for epidermal growth factor, exhibit a ligand-induced internalization that is followed by receptor breakdown in lysosomes along with ligands [4,5].

The normal turnover of recycling receptors could possibly be accounted for by an occasional mis-sorting of free receptor or receptor–ligand complexes to lysosomes, the latter perhaps as a result of a random failure of complexes to dissociate. Alternatively, the various proteolytic activities known to be present in prelysosomal compartments [6] may carry out receptor degradation, or at least the initiation thereof, in endosomal compartments. It is also possible that surface proteinases, such as the enzymes that degrade insulin [7] or the insulin receptor [8], or extracellular entities such as the kallikreins [9], may be the agents of receptor breakdown. We have accordingly studied LDL-receptor degradation in fibroblasts by a number of approaches. The acidotropic weak bases NH₄Cl and chloroquine had no inhibitory effect on

receptor degradation in the absence of ligand. Degradation of LDL receptors occurred at a reduced, but significant, rate at 18 °C, consistent with a degradation process that did not require endosome–lysosome fusion. Finally, the half-life of mutant LDL receptors that do not undergo internalization was similar to that of normal receptors, suggesting that endocytosis and recycling are not required for normal receptor breakdown.

MATERIALS AND METHODS

Materials

Human LDL [density (ρ) 1.019–1.063 g/ml] and human lipoprotein-deficient serum (LPDS) ($\rho > 1.25$ g/ml) were prepared by ultracentrifugation [10]. Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (MEM) and methionine-free MEM were purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Trans ³⁵S-label (³⁵S-label) from ICN Radiochemicals (Irvine, CA, U.S.A.) was the hydrolysate of *Escherichia coli* grown in the presence of H₂³⁵SO₄. 25-Hydroxycholesterol was obtained from Research Plus (Bayonne and Denville, NJ, U.S.A.).

Goat anti-mouse IgG (heavy- and light-chain-specific) was purchased from Cappel Laboratories (Malvern, PA, U.S.A.). IgG-C7 was prepared from hybridoma cells (American Type Culture Collection; CRL 1691) as described [11] and B3/25, a mouse monoclonal antibody to the human transferrin receptor, was generously given by Dr. I. S. Trowbridge (The Salk Institute for Biological Studies, San Diego, CA, U.S.A.).

Cells

Human skin fibroblasts, GM334BA (LDL receptor normal) and GM240BA (LDL receptor internalization-

Abbreviations used: LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; DMEM, Dulbecco's modified Eagle's medium; MEM, Eagle's minimal essential medium; ³⁵S-label, Trans ³⁵S-label (ICN Radiochemicals); *t*_{1/2}, half-life.

* To whom correspondence and reprint requests should be sent.

defective), were obtained from the Human Genetic Cell Repository (Camden, NJ, U.S.A.). Experiments were performed with actively growing cultures of cells grown in 60 mm-diameter Petri dishes in DMEM containing penicillin G (60 $\mu\text{g/ml}$), streptomycin sulphate (100 $\mu\text{g/ml}$) and 10% (v/v) heat-inactivated fetal-calf serum [11]. Before each experiment, cells were incubated in medium containing LPDS (5 mg of protein/ml) instead of fetal-calf serum, to up-regulate LDL receptors.

³⁵S pulse-chase experiments

Cells were incubated with ³⁵S-label in methionine-free MEM/LPDS and chased in DMEM/LPDS containing 200 μM unlabelled methionine, as indicated in the Figure legends. Receptor solubilization, immunoprecipitation and analyses by SDS/polyacrylamide-gel electrophoresis followed by fluorography were all performed as described previously [11].

Determination of half-life ($t_{1/2}$) values and statistical analysis of data

The $t_{1/2}$ values were obtained from best-fit single exponential curves fitted to data points using a weighted non-linear-regression program from Duggelby [12], and the parameters thus derived were compared by the Peritz' *F* test [13].

RESULTS AND DISCUSSION

LDL receptors recycle constitutively in both the presence or absence of LDL [14]. As we have previously reported [11], the $t_{1/2}$ of the LDL receptor was about 11 h as measured by [³⁵S]methionine pulse-chase experiments; this value was not affected by the presence of saturating concentrations of LDL in the medium (Fig. 1*a*). This result argues against the notion that a ligand-associated mis-sorting of receptors to lysosomes contributes to their degradation. The acidotropic weak bases NH_4Cl and chloroquine also had no inhibitory effect on the rate of receptor degradation in the absence of LDL (Figs. 1*b* and 1*c*). The presence of these agents, in the absence of ligand, had no effect on the number of LDL receptors on the cell surface, indicating that no intracellular accumulation of LDL receptors occurred and that recycling was not inhibited (results not shown). These results suggest that lysosomal enzymes, or proteinases of other acidic vesicles, are not responsible for the rate-limiting step(s) of receptor breakdown. In contrast, when LDL was present together with either of these two agents, receptor degradation was in fact accelerated (Figs. 1*b* and 1*c*). Under these conditions, recycling of receptors back to the cell surface was blocked, possibly because receptor-ligand complexes failed to dissociate; LDL receptors consequently accumulated inside the cell, where they were rapidly degraded (K. I. Grant, L. A. F. Casciola, G. A. Coetzee, D. A. Sanan, W. Gevers & D. R. van der Westhuyzen, unpublished work). Leupeptin, a cysteine-proteinase inhibitor, had no significant effect on receptor $t_{1/2}$ in the presence or absence of LDL (Fig. 1*d*), whereas ligand (LDL) degradation was inhibited by approx. 50% at the same concentrations (results not shown).

The degradation of the ligands of various receptors is severely retarded at 18 °C, owing to a block in endosome-lysosome fusion [15,16]. We have shown this to be true for LDL [17]. Degradation of LDL receptors at 18 °C, however, definitely took place at the approximately six

Fig. 1. Effect of NH_4Cl , chloroquine and leupeptin on the degradation of LDL receptors

On day 7 of cell culture, after a 24 h incubation with DMEM/LPDS to up-regulate LDL receptors, the cells were pulse-labelled for 16 h with 25 μCi of ³⁵S-label/ml. Cells were then chased for the indicated times in DMEM/LPDS medium containing 200 μM unlabelled methionine and supplemented with (a) no further additions (●, ○), (b) 10 mM- NH_4Cl (▼, ▽), (c) 75 μM -chloroquine (▲, △) or (d) leupeptin (150 $\mu\text{g/ml}$) (■, □) and in the absence (closed symbols) or presence (open symbols) of LDL (40 $\mu\text{g/ml}$). LDL receptors were immunoprecipitated from solubilized cell extracts with preformed immune complexes containing mouse monoclonal IgG-C7, and the labelled immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis and fluorography. Values are the mean values for duplicate dishes used in a single experiment but representative of at least three others. Duplicates never differed by more than 12% from the means. Receptor $t_{1/2}$ values in the absence of LDL were: control, 11.9 h; + NH_4Cl , 11.7 h; +chloroquine, 8.0 h (not significant, $P > 0.02$); +leupeptin, 11.6 h.

times lower rate expected from a process with a Q_{10} of about 2.5 ($t_{1/2} \approx 70$ h) (Fig. 2). Cell death or detachment did not apparently account for the loss of labelled receptors, since longer-lived transferrin receptors were degraded even more slowly ($t_{1/2} > 100$ h) in the same experiments. These results indicate that the degradation process for normal LDL receptors does not depend on the delivery of receptors to lysosomes through endosome-lysosome fusion.

Mutant LDL receptors found in GM240B cells are unable to cluster in coated pits and to be internalized as a result of a single amino acid substitution in the cytoplasmic tail of the molecule [18]. These receptors, which bind ligand and IgG-C7 antibody normally, remain randomly dispersed on the plasma membrane. The rate of turnover of these receptors was, nevertheless, the same as that of normal receptors (Fig. 3), suggesting that receptors are not degraded while undergoing internalization or recycling.

It should be mentioned that the degradation of LDL

Fig. 2. Degradation of LDL and transferrin receptors at 37°C and 18°C

Cells were up-regulated for 24 h, pulse-labelled for 7.5 h at 37°C with 40 μ Ci of 35 S-label/ml and then chased at the indicated temperatures. LDL and transferrin receptors were co-immunoprecipitated from solubilized cell extracts and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Values for 18°C incubations are means $\pm$ S.D. for triplicate dishes. Values at 37°C are of single dishes in the same experiment, representative of three others. LDL-receptor $t_{1/2}$ values were: at 18°C, 74 h (●); at 37°C, 11.1 h (○); transferrin receptor: at 18°C, 325 h (■); at 37°C, 48 h (□).

receptors was measured in these experiments by means of immunoprecipitation using the IgG-C7 monoclonal antibody. IgG-C7 recognizes the first N-terminal cysteine-rich repeat of the receptor which is not required for the binding of LDL [19,20]. Receptor disappearance may reflect total proteolysis of the molecule or more specific epitope losses, perhaps through the excision of the N-terminal region of the receptor. In any event we assume that loss of receptor immunoreactivity to IgG-C7 is associated with the pathway of LDL-receptor breakdown.

In summary, our results provide evidence that the initial rate-limiting steps of LDL-receptor degradation do not occur in lysosomes and are not associated with coated-pit-based endocytosis or receptor recycling.

This work was supported by grants from the South African Medical Research Council and from the University of Cape Town. K. Lyner and R. Liebenberg are thanked for excellent technical assistance.

REFERENCES

1. Brown, M. S. & Goldstein, J. L. (1986) *Science* **232**, 34–47
2. van der Westhuyzen, D. R. & Coetzee, G. A. (1985) in *Mononuclear Phagocytes: Physiology and Pathology* (Dean, R. T. & Jessup, W., eds.), pp. 91–111, Elsevier, Amsterdam
3. Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. & Schneider, W. J. (1985) *Annu. Rev. Cell Biol.* **1**, 1–39

Fig. 3. Comparison of the degradation of normal and internalization-defective mutant LDL receptors

Normal fibroblasts and internalization-defective cells (GM240BA cells) were up-regulated for 24 h and pulse-labelled for 7.5 h with 58 μ Ci of 35 S-label/ml. LDL receptors were immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Values are the means $\pm$ S.D. for triplicate dishes. LDL-receptor $t_{1/2}$ values were: normal, 11.2 h (□); GM240BA, 10.5 h (○).

4. Decker, S. J. (1984) *Mol. Cell. Biol.* **4**, 571–575
5. Dunn, W. A., Connolly, T. P. & Hubbard, A. L. (1986) *J. Cell Biol.* **102**, 24–36.
6. Diment, S., Leech, M. S. & Stahl, P. D. (1988) *J. Biol. Chem.* **263**, 6901–6907
7. Hammons, G. T., Smit, R. M. & Jarett, L. (1982) *J. Biol. Chem.* **257**, 11563–11570
8. Lipson, K. E., Kolhatkar, A. A. & Donner, D. B. (1988) *J. Biol. Chem.* **263**, 10495–10501
9. Drinkwater, C. C., Evans, B. A. & Richards, R. I. (1988) *Trends Biochem. Sci.* **13**, 169–172
10. Aulinskas, T. H., van der Westhuyzen, D. R. & Coetzee, G. A. (1983) *Atherosclerosis* **47**, 159–171
11. Casciola, L. A. F., van der Westhuyzen, D. R., Gevers, W. & Coetzee, G. A. (1988) *J. Lipid Res.* **29**, 1481–1489
12. Duggleby, R. G. (1981) *Anal. Biochem.* **110**, 9–18
13. Harper, J. F. (1984) *Comput. Biol. Med.* **14**, 437–445
14. Basu, S. K., Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1981) *Cell (Cambridge, Mass.)* **24**, 493–502
15. Dunn, W. A., Hubbard, A. L. & Aronson, N. N., Jr. (1980) *J. Biol. Chem.* **255**, 5971–5978
16. Kielian, M. C. & Cohn, Z. A. (1980) *J. Cell Biol.* **85**, 754–765
17. Aulinskas, T. H., Coetzee, G. A., Gevers, W. & Coetzee, G. A. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1551–1558
18. Davis, C. G., Lehrman, M. A., Russell, R. G. W., Brown, M. S. & Goldstein, J. L. (1986) *Cell (Cambridge, Mass.)* **5**, 15–24
19. van Driel, I. R., Goldstein, J. L., Südhof, T. C. & Brown, M. C. (1987) *J. Biol. Chem.* **262**, 17443–17449
20. Leitersdorf, E., Hobbs, H. H., Fourie, A. M., Jacobs, M., van der Westhuyzen, D. R. & Coetzee, G. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7912–7916