Two mutant low-density-lipoprotein receptors in Afrikaners slowly processed to surface forms exhibiting rapid degradation or functional heterogeneity

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Two distinct mutant low-density-lipoprotein receptors in South African Afrikaners exhibit retarded post-translational processing to mature forms. One mutation gives rise to cell-surface receptors that are subject to abnormally rapid degradation, whereas the other is associated with a functionally heterogeneous surface population degraded at a normal rate.

INTRODUCTION

Four functional classes of low-density-lipoprotein (LDL) receptor mutants have been identified by Brown & Goldstein (reviewed in [1]). Some mutant alleles at the LDL-receptor locus disrupt precursor synthesis (Class I), whereas others cause abnormal precursor processing (Class II), LDL binding (Class III) and receptor internalization (Class IV). These mutations have yielded important insights into the structure/function relationships of the different domains of the LDL receptor, such as structural requirements of the cytoplasmic tail for receptor internalization [2].

Familial hypercholesterolaemia (FH), an autosomal-dominant disorder caused by mutations in the LDL-receptor gene [1], is particularly prevalent in the South African Afrikaner population [3–5] with an estimated heterozygote frequency of 1:100 [4]. ‘Founder-gene’ effects, which occur in inbred but prolific populations such as the Afrikaner, have been proposed to account for this high incidence [4,5], and our previous studies of LDL receptor activity [6,7] and of a limited number of receptor-gene polymorphic restriction sites [8] are consistent with this concept. LDL receptors in 16 of the 17 Afrikaner families studied were classified [6,7] as being of the receptor-defective type (low but significant receptor activity) [9], and preliminary data [10] suggested the predominance of mutant genes coding for receptors which undergo retarded post-translational processing.

The aim of the present study was to characterize further the phenotypic expression of LDL receptors in cultured skin fibroblasts from selected Afrikaner FH homozygotes. Two mutant LDL receptors were distinguished on the basis of ligand- and antibody-binding studies and of receptor-turnover measurements. Both mutant receptors were processed slowly to their mature forms, which exhibited novel and contrasting behaviour.

MATERIALS AND METHODS

Surface binding of ¹²⁵I-labelled LDL or ¹²⁵I-labelled IgG-C7

Human skin fibroblasts were seeded (day 0) at 10⁵ cells

per 60 mm-diam. Petri dish in Dulbecco’s MEM (Flow Laboratories, Ayrshire, Scotland, U.K.), containing 10% (v/v) foetal-calf serum, and the medium was changed on day 3. On day 5, the medium was changed to one containing lipoprotein-deficient serum (LPDS) (5 mg of protein/ml) to up-regulate LDL receptor activity [7]. This medium was replaced with a fresh portion on day 6. On day 7, cells received 1.5 ml of ice-cold lipoprotein-deficient medium [7] and the indicated concentration of either ¹²⁵I-labelled LDL or ¹²⁵I-labelled IgG-C7. Human LDL ($\rho = 1.019\text{--}1.063\text{ g/ml}$) was prepared and radio-iodinated as described previously [7]. Monoclonal anti-LDL-receptor antibody was isolated from clonal hybridoma cells (American Type Culture Collection CRL 1691) and radioiodinated as described by Beisiegel *et al.* [11]. After incubation for 2 h at 4 °C with labelled ligand or antibody, cells were washed four times with Dulbecco’s phosphate-buffered saline (Ca²⁺- and Mg²⁺-free) containing 0.2% bovine serum albumin, followed by three washes with phosphate-buffered saline. The cells were solubilized in 1 M-NaOH and the total radioactivity bound to the cells was determined.

[³⁵S]Methionine pulse-chase experiments

Fibroblasts were seeded at 1.5×10^5 cells per 60 mm dish, and on day 2 the medium was replaced with lipoprotein-deficient medium. After 16 h, cells were preincubated in methionine-free Eagle’s MEM for 30 min at 37 °C, after which the cells were pulse-labelled at 37 °C with [³⁵S]methionine (40–50 $\mu\text{Ci/ml}$; Tran³⁵S-label; ICN Radiochemicals, Irvine, CA, U.S.A.). The medium was then changed to complete Dulbecco’s MEM/LPDS containing 200 μM unlabelled methionine, and the fibroblasts were incubated at 37 °C for various chase times. The cells were then washed and solubilized, and ³⁵S-labelled LDL receptors were immunoprecipitated with a preformed immune complex containing the monoclonal anti-LDL-receptor antibody, IgG-C7 [12]. Immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis (5–20% acrylamide gradient). The gels were enhanced by using salicylate [13], dried and exposed to X-ray film. The ³⁵S radioactivity in the

Abbreviations used: LDL, low-density lipoprotein; FH, familial hypercholesterolaemia; MEM, minimal essential medium.

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Fig. 1. Surface binding at 4 °C of ^{125}I -labelled LDL and ^{125}I -labelled IgG-C7 to normal and FH 1a fibroblasts

Cells from a normal subject (●) and FH 1a (○) were analysed for cell-surface binding at 4 °C of ^{125}I -labelled LDL (a) or ^{125}I -labelled IgG-C7 (b) as described in the Materials and methods section. Each value represents the average of duplicate incubations. The K_d values for ^{125}I -labelled LDL in normal and FH 1a cells were 2.4 and 3.0 μg of LDL protein/ml respectively, calculated by using the program 'Enzfitter' (Elsevier Biosoft), assuming ligand binding to one site and non-specific binding. Average K_d values from three experiments were $1.5 \pm 0.8 \mu\text{g}$ of LDL protein/ml in normal cells and $2.2 \pm 0.9 \mu\text{g}$ of LDL protein/ml in FH 1a cells. Maximum high-affinity binding of ^{125}I -labelled LDL in normal and FH 1a cells was 237 and 54 (23 % of normal) ng/mg of cell protein respectively. Maximum high-affinity binding of ^{125}I -labelled IgG-C7 was 41 and 33 (80 % of normal) ng/mg of cell protein respectively. In four separate experiments, the maximum high-affinity binding of ^{125}I -labelling IgG-C7 to FH 1a cells [56 ± 17 % (mean $\pm$ S.D.) of two receptor-normal controls] was 3.1 ± 0.5 times the high-affinity binding of ^{125}I -labelled LDL (18 ± 3 % of normal). FH 1b yielded quantitatively similar results to FH 1a for the relative binding of ^{125}I -labelled LDL and ^{125}I -labelled IgG-C7 in two separate experiments.

120 kDa precursor and 160 kDa mature receptor bands was quantified by densitometric scanning of the fluorograms.

RESULTS

Human skin fibroblasts from one pair of siblings (FH 1a and 1b), which previously exhibited the highest LDL receptor activity (~ 20 % of normal), were compared in the present study with cells (siblings FH 3a and 3b) of the one kindred in which virtually no activity (~ 2 % of normal) was previously detected [7]. On the basis of ten restriction-fragment length polymorphisms in the LDL-receptor gene, FH 1a and 1b were recently found to be homozygous for one LDL receptor allele, whereas FH 3a and 3b were homozygous for a second allele (E. Leitersdorf & H. H. Hobbs, personal communication).

Surface LDL receptors were characterized by ligand (LDL) and antibody (IgG-C7) binding. Cells from kindred-1 mutants showed a binding affinity for LDL ($K_d = 2.2 \mu\text{g}/\text{ml}$) which was similar to that of normal controls ($K_d = 1.5 \mu\text{g}/\text{ml}$), whereas the number of receptors in up-regulated cells, as assessed by high-affinity LDL binding, was approx. 20 % of normal (Fig. 1a). Unexpectedly, when receptors were then assessed on the basis of the high-affinity binding of IgG-C7 (a monoclonal antibody which recognizes an epitope distinct from the LDL-binding site), kindred-1 mutant cells showed a significantly greater number of receptor molecules (50–80 % of normal) (Fig. 1b). In these experiments, receptor number was assessed on the basis of high-affinity binding of ^{125}I -LDL or ^{125}I -IgG-C7. Total binding was corrected for low-affinity values, which were similar in normal and kindred-1 cells. Specificity of the high-affinity component was indicated by the fact that down-regulation of the LDL receptor by sterols [11] decreased total binding of ^{125}I -LDL or ^{125}I -IgG-C7 by an amount equal to the high-affinity binding (results not shown). In all experiments with kindred-1 cells, the maximum high-affinity binding of IgG-C7 was several-fold higher than that of LDL. These results suggest the presence of one population comprising fully active receptors possessing normal high affinity for LDL, and a second consisting of inactive receptors which fail to bind LDL but which can nevertheless be recognized immunologically. Kindred-3 homozygous cells, in contrast, showed extremely low amounts of surface receptor protein (< 5 % of normal) measured either with LDL or with IgG-C7 [7].

To assess the possible effect of synthesis rates on receptor numbers, initial rates of [^{35}S]methionine incorporation into immunoprecipitable LDL receptors were measured. In the kindred-1 mutant, the rates of LDL-receptor synthesis (measured relative to the synthesis of total protein or another surface protein, the transferrin receptor) were 80–90 % of normal. Cells from kindred-3 mutants synthesized receptor protein at slightly decreased rates (~ 60 % of normal), indicating that their receptor-negative phenotype was not the result of a 'null' allele failing to express a protein product.

Post-translational processing of LDL receptors was studied by [^{35}S]methionine pulse-chase experiments. The precursor form of the normal receptor (apparent molecular mass = 120 kDa) was rapidly and quantitatively processed ($t_{1/2}$ approx. 15 min) to a 160 kDa mature form by the addition of oligosaccharides (Fig. 2a) [12,14]. The newly synthesized precursor in the kindred-1 mutants (Fig. 2b) had an apparently normal molecular mass (120 kDa), indicating that the mutation caused no large deletion or insertion in the protein. However, the

Fig. 2. Processing of LDL receptors in normal and FH homozygous fibroblasts

Up-regulated fibroblasts from a normal subject (a) and from FH 1a (b) and 3b (c) were pulse-labelled with [35 S]-methionine for 1 h (normal and FH 3b) or 2 h (FH 1a), and the radioactivity in the 120 kDa precursor (○) and 160 kDa mature (●) LDL receptors was quantified as described in the Materials and methods section. Values are expressed as a percentage of the maximum total 35 S incorporation. The band above the 160 kDa band is a non-specific contaminant also present in immunoprecipitates from down-regulated cells. FH 1b and FH 3a yielded processing kinetics virtually identical with those of FH 1a and FH 3b respectively (results not shown).

conversion of precursor into mature 160 kDa receptor, which accompanies transport to the Golgi complex and maturation of core sugars, was severely retarded. In cells from kindred 3, processing of apparently normal precursors to normal-sized mature receptors occurred at a markedly faster rate than in kindred-1 cells, although still somewhat slower than in normal cells (Fig. 2c).

Another feature which distinguished the mutant receptors of these two kindreds from each other was their rate of degradation. The half-life of the mature receptors in kindred-1 cells and in normal cells (Fig. 3) was about 8 h, a value similar to those reported previously for the normal LDL receptor [5,15]. In kindred 3, on the other hand, mature mutant LDL receptors were turned over extremely rapidly, with a half-life of about 1.5 h (Fig. 3). The loss of label from the mature receptors in kindred 3

Fig. 3. Degradation of 35 S-labelled mature receptors in cells from a normal subject and from FH 1a and FH 3b

Up-regulated fibroblasts from a normal subject and from FH 1a and FH 3b were preincubated in methionine-free Eagle's MEM for 30 min at 37 °C, after which the cells were pulse-labelled at 37 °C with [35 S]methionine for 4 h at 20 μ Ci/ml (Normal and FH 1a) or 2 h at 60 μ Ci/ml (FH 3b). The medium was then changed to complete Dulbecco's MEM/LPDS (containing 200 μ M-methionine), and the fibroblasts were incubated at 37 °C for various times until virtually none of the 35 S label remained in the precursor (120 kDa) band. This was assumed to be the 'zero' chase-time point (normal and FH 1a, 11.5 h; FH 3b, 3 h). After the subsequent chase period, immunoprecipitation, SDS/polyacrylamide-gel electrophoresis and fluorography of labelled receptors were performed as described in the Materials and methods section. The $t_{1/2}$ values (means $\pm$ S.E.M.) for LDL-receptor degradation (determined by non-linear regression) were: normal, 8.9 ± 1.4 h; FH 1a, 7.5 ± 1.4 h; FH 1b, 9.0 ± 0.9 h (results not shown); FH 3a, 1.5 ± 0.2 h (results not shown); FH 3b, 1.6 ± 0.2 h.

apparently was not due to secretion or epitope denaturation, since no secreted receptor could be detected in the medium, and since a polyclonal anti-LDL-receptor antibody did not precipitate any receptors not recognized by IgG-C7 (results not shown). Pronase and neuraminidase treatments were used to determine whether mature receptors in kindred-3 cells were trapped and degraded intracellularly before insertion into the plasma membrane, or whether they were transported to the surface to

Fig. 4. Sensitivity of LDL receptors in normal and FH 3b cells to treatment with neuraminidase and Pronase

For lanes 1 and 2, normal fibroblasts were pulse-labelled with [35 S]methionine for 30 min, followed by a 30 min chase period. 35 S-labelled LDL receptors were then immunoprecipitated, and the immunoprecipitates were incubated overnight at 4 °C in the absence or presence of neuraminidase (0.8 unit/ml; Calbiochem-Behring Diagnostics) [22]. For lanes 3–15, cells from the indicated subject were pulse-labelled with [35 S]methionine (84 μ Ci/ml) in methionine-free medium for 60 min at 37 °C, followed by a chase for 60 min in medium containing 200 μ M unlabelled methionine. The chase medium was removed and cells were washed and incubated at 37 °C in the absence or presence of neuraminidase (0.05 unit/ml) for 30 min [17] or Pronase (20 μ g/ml; Boehringer-Mannheim) [16] for 20 min. The cells were then washed and solubilized, and 35 S-labelled LDL receptors were immunoprecipitated. Immunoprecipitates 1–15 were subjected to SDS/polyacrylamide-gel electrophoresis (10–20%-acrylamide gradient), after which the gel was enhanced, dried and exposed to X-ray film.

be degraded at a later stage. Pronase is known to degrade effectively surface LDL receptors [16], whereas neuraminidase causes a small but significant decrease in their apparent molecular mass on SDS/polyacrylamide-gel electrophoresis through removal of sialic acid residues [17]. The mature mutant receptor was almost entirely destroyed by Pronase during incubation of intact cells at 37 °C (Fig. 4; cf. lanes 12 and 13 with 14 and 15). Neuraminidase decreased the apparent molecular mass of all of the mature mutant receptors (Fig. 4; cf. lanes 6 and 7 with 8 and 9). Therefore the kindred-3 mutant receptors were exposed on the cell surface during their life-span.

DISCUSSION

We describe in this report the phenotypic expression of LDL-receptor mutations in two South African Afrikaner kindreds. The gene products of both mutations are slowly processed to mature surface forms, each exhibiting a distinct behaviour not characteristic of any other known LDL-receptor mutant.

An important observation regarding the kindred-1 mutation is that cells homozygous for a single mutant allele apparently display a functionally heterogeneous receptor population on their surfaces. Thus a single gene product is apparently present in one of two forms: an active receptor species which binds LDL with normal high affinity, and an inactive form which fails to bind LDL but which interacts with IgG-C7. Since the mature receptors are apparently intact and normally glycosylated, as judged by SDS/polyacrylamide-gel electrophoresis, a possible explanation for this behaviour is one involving alternative conformations of receptor mol-

ecules. Abnormal folding or denaturation of receptor precursors may also be responsible for the delay in transport and processing (reviewed in ref. [18]). With respect to this retarded processing, the kindred-1 mutant receptor can be grouped with the so-called 'Class II variants', which differ from the typical Class II mutants in which processing is virtually absent [10]. These variants include the LDL receptors in the WHHL rabbit and a homozygous patient, FH 563 [19]. Both these receptors have small deletions in the cysteine-rich, highly disulphide-bonded binding domain, which may lead to incorrect folding and hence delayed transport of the protein to the Golgi complex [19]. The slowly processed receptors in kindred-1 cells may be transported to the cell surface after various degrees of conformational 'trial-and-error'. We postulate that some of these mature receptors bind LDL normally, whereas others remain conformationally abnormal. This differs from the idea that in the Class II variants a homogeneous receptor population binds LDL inefficiently [10]. Interestingly, the entire receptor population in kindred-1 cells, including the large proportion of inactive receptors, is turned over with an apparently normal half-life (Fig. 3).

The kindred-3 mutant receptor exhibits a distinctive phenotype. Mature receptors are readily formed and reach the cell surface, but undergo extremely rapid degradation, which can account for the very low surface receptor activity in these cells. This is the first report of a naturally occurring LDL-receptor mutation resulting in greatly accelerated turnover of cell-surface receptors. The only precedents for rapid degradation of mature LDL receptors are constructed mutations where the whole epidermal-growth-factor precursor-like domain, or only the cysteine-rich repeats thereof, have been deleted by site-directed mutagenesis [20]. These mutant receptors are rapidly degraded in transfected cells in the presence of the ligand very-low-density lipoprotein, but not in its absence. The dissociation of the ligand from the mutant receptor in the acidic endosomal compartment is prevented, presumably because the receptor is unable to undergo the proper acid-dependent conformational changes. Recycling seems to require a relatively stable receptor structure to allow repeated passage through the acidic endosome without irreversible denaturation of receptors taking place [10]. The mutant receptors of kindred 3 may have a mutation in one of the cysteine-rich regions which renders them unstable to continual recycling and results in rapid degradation. Whatever the underlying mechanism, the phenotypic expression of the LDL-receptor mutation in this kindred represents a new class of defects.

This report represents the first description of the cellular events in the phenotypic expression of two distinct LDL-receptor mutations in South African Afrikaners. The novel LDL-receptor mutations reported here also have particular relevance to two processes of great current interest in cell biology, namely intracellular transport and sorting of secretory or cell-membrane proteins [18] on the one hand, and the turnover of receptor molecules [21] on the other.

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