Comparison of Glucosylated Low Density Lipoprotein with Methylated or Cyclohexanedione-treated Low Density Lipoprotein in the Measurement of Receptor-independent Low Density Lipoprotein Catabolism

URS P. STEINBRECHER, JOSEPH L. WITZTUM, Y. ANTERO KESANIEMI, and RICHARD L. ELAM, *Division of Metabolic Disease, Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, California 92093*

ABSTRACT We previously showed that glucosylation of lysine residues of low density lipoproteins (LDL) blocks high-affinity degradation by cultured human fibroblasts, and markedly slows LDL turnover in guinea pigs. The present studies were done to evaluate glucosylated (GLC) LDL as a tracer of receptor-independent LDL catabolism, and to compare it with two other modified LDL, methylated (MET) LDL, and cyclohexanedione (CHD)-treated LDL, which have been used previously for this purpose. Glucosylation of LDL did not affect receptor-independent degradation in vivo, as the turnover of GLC-LDL and native LDL were similar in the LDL receptor-deficient, Watanabe heritable hyperlipidemic rabbit. Each modified radiolabeled LDL preparation was injected into eight guinea pigs, and fractional catabolic rates (FCR) determined. The FCR of GLC-LDL (0.024±0.005 h^{-1}; SD) was similar to that of MET-LDL (0.023±0.006 h^{-1}), and ~22% of that of native LDL (0.105±0.02 h^{-1}). The FCR of CHD-LDL was greater than that of the other modified LDL, and it varied depending on how soon after preparation the CHD-LDL was injected: when used within 2 h of preparation, the mean FCR was 0.044±0.007 h^{-1} (n = 4); when used after overnight dialysis at 4°C, the mean FCR was 0.082±0.03 h^{-1} (n = 4). This suggests that CHD-LDL overestimates the amount of LDL degraded by receptor-independent pathways, perhaps because the CHD modification is spontaneously reversible. The present studies indicate that GLC-LDL is a useful tracer of receptor-independent LDL catabolism in animals.

INTRODUCTION

Degradation of low density lipoproteins (LDL) by cultured fibroblasts occurs via a high affinity, saturable "LDL pathway" and also by low affinity, nonsaturable, "receptor-independent" processes (1, 2). Chemical modification of LDL by reductive methylation (MET)[1] or treatment with cyclohexanedione (CHD) inhibits binding and uptake by the LDL receptor pathway in cultured cells. If these chemical modifications block only receptor-mediated catabolism, then it should be possible to estimate the fraction of total LDL catabolism that occurs via the LDL receptor in vivo by measuring the difference between the catabolic rate of native and modified LDL (3, 4). Unfortunately, several drawbacks are associated with the methods currently used, in that the CHD modification is spontaneously reversible in physiologic solutions (4–6) and hence might overestimate receptor-independent catabolism, while MET-LDL appears unsuitable for use in humans as it is cleared rapidly from plasma by an as yet undefined mechanism (6).

We have previously reported that glucosylation (GLC) of lysyl residues of LDL blocks high affinity binding and degradation by cultured human fibroblasts and markedly slows LDL turnover in guinea pigs

Dr. Steinbrecher is a Fellow of the Medical Research Council of Canada, and Dr. Kesaniemi is a Visiting Scientist from the 2nd Department of Medicine, University of Helsinki, Finland. Address all correspondence to Dr. Steinbrecher.

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[1] *Abbreviations used in this paper*: apo, apolipoprotein; CHD, cyclohexanedione; FCR, fractional catabolic rate; GLC, glucosylated (LDL); MET, methylated (LDL); NZW, New Zealand white; WHHL, Watanabe heritable hyperlipidemic (rabbit).

(7). Because GLC-LDL is stable, and therefore might be preferable to CHD-LDL as a tool for measuring receptor-mediated catabolism, further studies to define the completeness and specificity of the blockade of receptor-mediated catabolism produced by glucosylation seemed appropriate. We undertook the present studies to compare the degree to which the various modifications inhibited LDL catabolism in cultured normal human fibroblasts, and in vivo in guinea pigs. In addition, to determine if glucosylation of LDL affected LDL receptor-independent processes, we compared the turnover of GLC-LDL with that of native LDL in the receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbit.

METHODS

Isolation and modification of lipoproteins. Human LDL (d = 1.019–1.063 g/ml) and lipoprotein-deficient serum were prepared from pooled normal human plasma by sequential ultracentrifugation as previously described (8). In preliminary studies, we found that in guinea pigs there was no difference in the clearance of human and guinea pig LDL, and no difference in the clearance of the corresponding glucosylated LDL preparations. On this basis, human LDL was used for the studies in guinea pigs. In rabbits, human LDL is cleared at a different rate than homologous LDL (9), therefore, only rabbit LDL was used for studies in this species. Rabbit LDL was isolated in the density range, 1.025–1.063, from a New Zealand white (NZW) rabbit (Red Beau Farms, Redlands, CA). Radioiodination was carried out by a modification (10) of the iodine monochloride method by using carrier-free $Na^{125}I$ or $Na^{131}I$ (Amersham Corp., Arlington Heights, IL). After extensive dialysis against phosphate-buffered saline with 0.01% EDTA, pH 7.35 (PBS), >98.5% of radioactivity was precipitable by 10% trichloroacetic acid (TCA). Less than 6% of the radioactivity was extractable into organic solvents. Specific activities of the labeled LDL preparations ranged between 74 and 180 cpm/ng. The same ^{125}I-LDL or ^{131}I-LDL preparations were used to prepare all the modified LDL in a given set of experiments.

GLC-LDL was prepared under sterile conditions by incubating LDL (final concentration 2 mg/ml) for 5 d at 37°C with 80 mM glucose (Mallinckrodt Inc., Paris, KY) and 200 mM $NaCNBH_3$ (Aldrich Chemical Co., Inc., Milwaukee, WI) in PBS followed by extensive dialysis against PBS (7). These conditions resulted in glucosylation of between 45 and 60% of lysine residues of LDL as determined by amino acid analysis (7) or by trinitrobenzenesulfonic acid assay (11). Recovery of LDL protein was >90% after glucosylation and dialysis. After incubation, >98% of the radioactivity remained precipitable by 10% TCA, and >88% was recoverable in the LDL density range upon ultracentrifugation. The lipid composition and the cholesterol/protein ratio of the ultracentrifugally reisolated control and glucosylated LDL were unchanged. Electrophoresis in the presence of sodium dodecyl sulfate with a 3–7% polyacrylamide gradient gel showed a very slight reduction in migration of GLC-LDL apolipoprotein (apo)-B as compared with native LDL apo-B; this is consistent with the known ability of carbohydrate to interact with polyacrylamide to slow migration. MET-LDL was prepared as previously described (12), by sequential addition with gentle stirring of 10 aliquots of formaldehyde over a 1-h period to LDL in borate buffer, pH 9.1, with addition of 1 mg of $NaBH_4$ at the start of the reaction, and again 30 min later. The extent of methylation of lysine residues of MET-LDL as determined by the trinitrobenzenesulfonic acid method was >85%. CHD-LDL was prepared by incubation of LDL for 2 h at 37°C with 0.15 M CHD in 0.2 M borate buffer, pH 8.1 (13). In one set of experiments, unreacted CHD was removed by dialysis for 14 h against PBS at 4°C, while a subsequent set was done using CHD-LDL within 2 h of preparation, after chromatography on Sephadex G-25 to remove unreacted CHD.

Cell culture studies. Skin fibroblasts were obtained from a preputial biopsy of a normal infant (B.B.), and maintained as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells taken between the 6th and 12th passage were seeded onto 35-mm tissue culture plates and were used for experiments when cells reached ~70–80% confluence (80–160 μg of cell protein/dish). To stimulate expression of LDL receptors, the medium was changed to Dulbecco's modified Eagle's medium containing 5% lipoprotein-deficient serum 24 h before use in experiments. Cells were harvested after 12–20 h of incubation with radiolabeled LDL. The content of TCA-soluble, noniodide radioactivity in the medium was used to calculate the amount of LDL degraded (14). After removal of the media, cells were washed with PBS, dissolved in 0.25 M NaOH, and then aliquots were taken for protein determination by the method of Lowry (15).

Turnover studies. Male Hartley guinea pigs weighing between 400 and 600 g were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). The animals were fed Wayne guinea pig chow (ICN Nutritional Biochemicals, Cleveland, OH) ad lib., and KI was added to the drinking water during the studies. Two differently labeled, modified LDL preparations were injected simultaneously into an exposed external jugular vein. Serial blood samples, each 0.2 ml in volume, were then obtained over a 90-h period by cardiac puncture with a 25-gauge needle, with the animals lightly anesthetized with ether. To obtain additional evidence that glucosylation does not affect receptor-independent LDL catabolism, we compared the turnover of rabbit GLC-LDL and native rabbit LDL in a 2.5-kg NZW rabbit and in a 2.7-kg, 2-yr-old WHHL rabbit obtained from Professor Y. Watanabe, (Kobe, Japan). The radiolabeled LDL preparations were injected into a marginal ear vein, and blood samples obtained from a different ear vein with the animals immobilized in restraint cages without anesthesia. All blood samples were anticoagulated with solid EDTA, plasma was separated by centrifugation at 3,000 rpm, and aliquots were counted on a double-channel gamma spectrometer. Two exponential equations were fitted to each plasma decay curve by using an interactive curve-peeling program (W. F. Beltz and T. E. Carew Unpublished method.) on a VAX/VMS computer (Digital Equipment Corp., Marlboro, MA). Fractional catabolic rates (FCR) were computed as the reciprocal of the area under the normalized radioactivity-time curve (16). Significance of differences between means of FCR for each type of LDL modification was assessed by using the nonpaired t test with level of significance assessed by two-tailed probability tables.

RESULTS

Cell culture studies. In agreement with previous reports (7, 12, 13), high affinity degradation by normal fibroblasts was inhibited with each type of modified

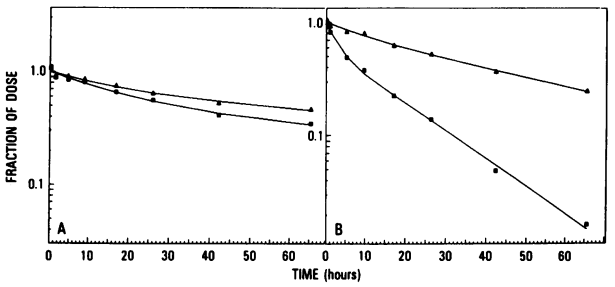

FIGURE 1 Plasma radioactivity decay curves of ^{131}I normal rabbit LDL (■) and ^{125}I rabbit GLC-LDL (▲) after simultaneous injections of 1 μCi of the ^{131}I label and 4 μCi of the ^{125}I label into a WHHL rabbit (A) and a normal NZW rabbit (B). Serial blood samples were drawn from an ear vein. The radioactivity in each sample is expressed as a fraction of the initial dose, which was calculated by extrapolation of the plasma decay curve to zero time. The curves were analyzed as described in Methods to obtain FCR values, for native LDL and GLC-LDL, respectively, which were 0.0095 h^{-1} and 0.0085 h^{-1} in the WHHL rabbit, and 0.086 h^{-1} and 0.021 h^{-1} in the normal NZW rabbit.

LDL. The degradation rates for GLC-LDL and MET-LDL were consistently <2% of the rate for native LDL, whereas values for CHD-LDL ranged from 3 to 20% of the rate for native LDL; the higher results were obtained in experiments involving 20-h incubations and may have reflected partial reversal of the CHD modification during the incubation.

Animal studies. In the receptor-deficient WHHL rabbit, the FCR of GLC-LDL was very close to that of native LDL (Fig. 1A), indicating that glucosylation of LDL does not significantly alter receptor-independent catabolism in vivo.

Examples of typical paired turnover studies in guinea pigs comparing GLC-LDL with MET-LDL, MET-LDL with CHD-LDL, and GLC-LDL with CHD-LDL are shown in Fig. 2. Individual FCR values for each modified LDL are shown in Fig. 3 together with values for native human LDL obtained in prior experiments under identical conditions. The mean FCR of GLC-LDL (0.024 h^{-1}) was essentially identical to that of MET-LDL (0.023 h^{-1}). The FCR of CHD-LDL varied depending on how soon after preparation the material was injected: CHD-LDL used within 2 h of preparation had a mean FCR of 0.044 h^{-1}, whereas CHD-LDL used after dialysis for 14 h at 4°C had a mean FCR of 0.082 h^{-1}. Analysis of these data using the *t* test reveals that the difference between the mean FCR of CHD-LDL used within 2 h of preparation and that of MET-LDL or GLC-LDL is significant ($P < 0.001$). The mean FCR of CHD-LDL used after 14 h of dialysis was greater ($P < 0.05$) than that of CHD-LDL used within 2 h of preparation but was not different ($P > 0.1$) from the mean FCR of native LDL.

Estimation of the proportion of total LDL catabolism due to receptor-dependent processes in the guinea pig by using the mean FCR results shown in Fig. 3 yields values of 78–79% for the GLC-LDL and MET-LDL tracers. In contrast, the CHD-LDL tracer used within 2 h of preparation gives a value of only 60% for receptor-mediated catabolism. The turnover results in the normal NZW rabbit (Fig. 1B), using GLC-LDL as the tracer of receptor-independent catabolism, yield a value of 75% for the ratio of receptor-dependent to total LDL catabolism. This is similar to the value reported by Bilheimer and colleagues (9) in NZW rabbits, with MET-LDL as the tracer of receptor-independent catabolism.

DISCUSSION

If a particular modified LDL preparation is to be of value as a tracer of receptor-independent LDL catabolism, it must be shown that the modification is irreversible, that uptake via the LDL receptor is totally blocked by the modification, and that the modification does not alter catabolism of LDL by other pathways.

Nonenzymatic glucosylation of LDL in the presence

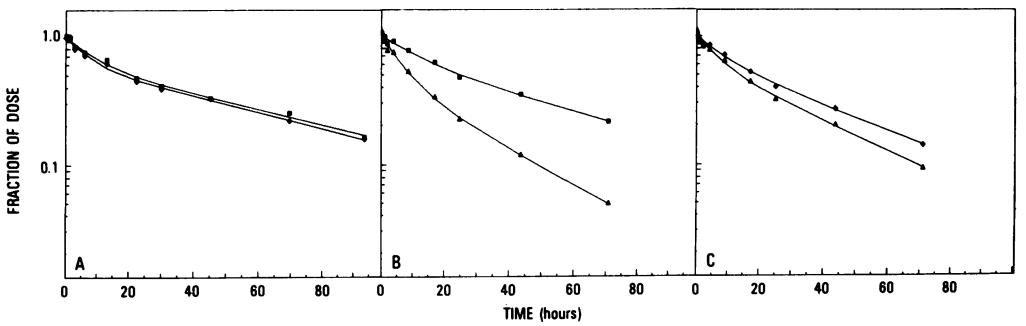

FIGURE 2 Representative plasma radioactivity decay curves for human GLC-LDL (♦), MET-LDL (■), and CHD-LDL (▲) in guinea pigs. Approximately 1 μCi of the ^{131}I label and 4 μCi of the ^{125}I label were injected simultaneously into an exposed external jugular vein, and then serial blood samples were collected by cardiac puncture. Panel A shows that the curves for ^{131}I-MET-LDL and ^{125}I-GLC-LDL are nearly superimposable, while the curves shown in B and C indicate that the clearance of CHD-LDL used within 2 h of preparation is variable, but greater than that of MET-LDL (B) or GLC-LDL(C).

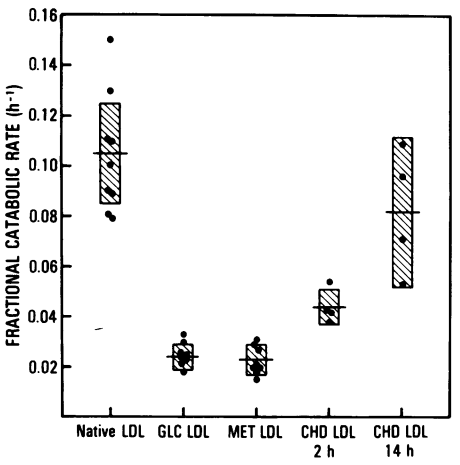

FIGURE 3 FCR for native human LDL and modified LDL preparations in guinea pigs. Each point represents turnover results in one animal. The hatched bars indicate standard deviation. The values for native LDL were obtained in previous studies with identical conditions to those described. A total of eight animals were injected with each modified LDL from two different preparations of freshly prepared modified LDL. The data for the MET-LDL and GLC-LDL turnovers from the two experiments are combined. In one experiment, CHD-LDL was used within 2 h of preparation, after gel filtration chromatography to remove unreacted CHD (CHD-LDL 2 h), and a second experiment was done with CHD-LDL after overnight dialysis at 4°C against PBS (CHD-LDL 14 h). Statistical analysis using the unpaired t test and two-tailed probability tables indicates that the difference between the mean FCR of CHD-LDL at 2 h and either MET-LDL or GLC-LDL is significant ($P < 0.001$). The value for CHD-LDL at 14 h is greater ($P < 0.05$) than that for CHD-LDL at 2 h, but not different from native LDL ($P > 0.1$).

of cyanoborohydride is evidently irreversible, in that the glucitol lysine product is stable even to boiling in 6 N HCl for 24 h (7). There are at least three lines of evidence that indicate that uptake via the LDL receptor is completely blocked. First, experiments using cultured normal human fibroblasts show no high-affinity, saturable LDL degradation when greater than one-third of lysine residues are glucosylated (7). In the present studies, the extent of glucosylation was 45–60%, and every preparation was checked in cultured fibroblasts to confirm that interaction with the LDL receptor was blocked. A second approach was the demonstration that the turnover of GLC-LDL is identical to that of MET-LDL in guinea pigs. Methylation of greater than one-third of lysine residues of LDL results in inhibition of receptor-mediated degradation by fibroblasts similar to that with GLC-LDL (12). The observation that two different modifications give identical results for receptor-independent catabolism strengthens confidence in both methods. Third, in other studies (17), we have found that the turnover of GLC-LDL in human subjects (FCR of 0.11 d^{-1}, $n = 4$) is comparable with values reported for native LDL turnover in patients with homozygous familial hypercholesterolemia (18). Thus, there is evidence in vitro, in animals, and in humans, that glucosylation effectively blocks the ability of LDL to interact with the LDL receptor.

To validate GLC-LDL as a tracer of receptor-independent LDL catabolism, it must also be demonstrated that GLC-LDL is handled in an identical manner to native LDL by the receptor-independent pathway, i.e., that the modification blocks only receptor-dependent processes and not others. To ascertain if glucosylation of LDL affects receptor-independent catabolism in vivo, we compared the turnover of native LDL and GLC-LDL in the WHHL rabbit, a model of homozygous familial hypercholesterolemia (19, 22). These mutant rabbits have very little or no detectable LDL receptor activity and hence must degrade LDL exclusively by receptor-independent pathways. We found that the turnover rates of native LDL and GLC-LDL were very similar in these animals, indicating that glucosylation did not alter receptor-independent LDL catabolism. Similar observations have been reported with MET-LDL in the WHHL rabbit (9).

We found that the turnover of CHD-LDL in guinea pigs was consistently greater than that of either GLC-LDL or MET-LDL. This might be due to enhanced clearance by receptor-independent mechanisms of CHD-LDL as compared with the other two tracers (6). However, we feel it is most likely due to slow spontaneous reversal of the CHD modification, as was first demonstrated by Mahley et al. (4), who found that 53% of [^{14}C]CHD dissociated from [^{14}C]CHD-LDL after 24 h of incubation in serum at 37°C, and that such incubation led to a progressive restoration of binding activity toward the LDL receptor. This observation has been confirmed by Slater et al. (6). Our results showing an increase in turnover of CHD-LDL after overnight dialysis against PBS, when compared with that of freshly prepared CHD-LDL, are consistent with time-dependent reversal of the CHD modification. In addition, we found that even CHD-LDL used within 2 h of preparation was cleared more rapidly than MET-LDL or GLC-LDL in guinea pigs; similar results have been described by Slater et al. (6) with CHD-LDL and MET-LDL in rabbits. Kinetic modeling of our data indicates that the difference in FCR we observed between CHD-LDL and GLC-LDL or MET-LDL can be entirely explained by reversal of the CHD-modification if the rate of reversal in vivo is the same as that reported in vitro (4), and if the regenerated LDL is then catabolized at the same rate as native LDL (T. E. Carew, personal communication).

In summary, these data suggest that both MET-LDL and GLC-LDL are valuable tools for measuring receptor-independent LDL catabolism in animals. Very similar values for receptor-independent LDL catabolism are obtained when the two tracers are compared

in guinea pigs and rabbits. In addition, MET-LDL has been successfully used in measuring receptor-independent LDL catabolism in rats (4, 23) and monkeys (4). However, when labeled MET-LDL was injected into humans, a very rapid plasma clearance was noted (6). In contrast, we have observed a slow monoexponential decay of plasma radioactivity when GLC-LDL was injected into normal subjects (17), suggesting that GLC-LDL should be a useful tracer of receptor-independent catabolism in humans. We have made simultaneous measurements of the turnover of GLC-LDL and native LDL in four normal individuals, and found that in every case the FCR of GLC-LDL was 20% of that of native LDL, indicating that 80% of total LDL catabolism in normal humans occurs via the LDL receptor pathway (17). However, in recent studies involving diabetic subjects, we have observed several cases where, after an initial phase of slow decay lasting 4–10 d, an abrupt increase in clearance of GLC-LDL occurred. This phenomenon is currently being investigated and may have an immunologic basis. Although we have noted no adverse effects in any of our human subjects, caution should be exercised with the use of GLC-LDL in human studies until this problem has been clarified.

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