papers on methodology

Rapid fluorometric assay of LDL receptor activity by DiI-labeled LDL

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Abstract DiI-LDL (3,3'-dioctadecylindocarbocyanine-low density lipoprotein) has been extensively used in morphological and microscopic studies of receptor-mediated metabolism of LDL in many cell lines. To date the use of this fluorescent probe in a quantitative assay of LDL receptor activity has not been widely used in studies with multiple samples due to the lack of a practical method for quantitatively recovering cell-associated DiI. Therefore, detection by ^{125}I-labeled LDL has remained the method of choice for assaying LDL receptor activity rapidly and reliably. In this paper, we describe a rapid, simple, and nonradioactive assay of LDL receptor activity using DiI-LDL. The increased sensitivity of this method was achieved by modifications to the labeling procedure of LDL and to the extraction of DiI from cells for subsequent fluorescence determination. These modifications did not affect the affinity of DiI-LDL toward HepG2 cells, and the assay was easily adapted to a rapid screen for LDL receptor modulators in this cell model.—**Stephan, Z. F., and E. C. Yurachek.** Rapid fluorometric assay of LDL receptor activity by DiI-labeled LDL. *J. Lipid Res.* 1993. **34:** 325–330.

Supplementary key words HepG2 cells • lovastatin • 25OH-cholesterol

Elevated serum cholesterol, especially LDL cholesterol, has been well established as a major risk factor in the development of atherosclerosis (1). Because the up-regulation of LDL receptor has been shown to play a pivotal role in enhancing the clearance of LDL cholesterol (2) from circulation and reducing the risk of atherogenesis (3), the search for novel, safe, and efficacious lipid-lowering agents working by this mechanism has rapidly intensified.

To date, a wide variety of compounds such as cholesterol synthesis inhibitors (lovastatin, ketoconazole), hormones (thyroxin, insulin, estradiol), growth factors (PDGF, EGF), and others (calmodulin antagonists, Ca^{2+} channel blocker, β-blocker) have been known to up-regulate LDL receptor activity in cells by various mechanisms. So far, the detection methods used to screen compounds have relied primarily on ^{125}I-labeled LDL which, in spite of its reliability and high sensitivity, adds to a growing concern about waste management, disposal cost, and personal safety.

Detailed studies of LDL interaction with its cellular receptor using the fluorescent probe DiI have been known for at least a decade (4, 5). However, this technique has been primarily limited to morphological and microscopical studies of the LDL receptor (6–8). Furthermore, detection by ^{125}I-labeled LDL has been the method of choice for the rapid quantitation of receptor -mediated LDL metabolism since it was first introduced by Brown and Goldstein (9, 10) despite the development of several new methods, such as enzyme-linked immunoreceptor assay (11), immunoblotting (12), laser spectrofluorometry (13), photobleaching (14), electroblotting and detection with biotinylated (15), or gold-LDL (16). Even though all of these methods were highly sensitive, they proved to be too labor-intensive and therefore too costly and impractical to be applied to studies with multiple samples. In this paper, we describe a simple, rapid, and nonradioactive method using DiI-LDL for the quantitative measurement of LDL receptor activity. This method could easily be adapted to a high through-put screen of compounds modulating LDL receptor activity in cells.

MATERIALS AND METHODS

Isolation and labeling of lipoprotein

Human LDL was prepared from normolipemic plasma by sequential ultracentrifugation (17). Briefly, VLDL was first discarded after spinning plasma at 39,000 rpm for 18 h using a fixed-angle rotor (Ti 50) and Model L8-M ultracentrifuge (Beckman, Palo Alto, CA). Subsequently, LDL with a density range of 1.020–1.040 g/ml was prepared from VLDL-free plasma layered with 1.063 g/ml

Abbreviations: DiI, 3, 3'-dioctadecylindocarbocyanine; LDL, low density lipoprotein; VLDL, very low density lipoprotein; FBS, fetal bovine serum; HSA, human serum albumin; PBS, phosphate-buffered saline.

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KBr solution and spun at 39,000 rpm for 24 h. LDL was extensively dialyzed against normal saline for 24 h, filter-sterilized (0.45 μm, Waters Millex HV units) and its final protein concentration was determined as described by Bradford (18), and adjusted to 1 mg/ml.

LDL was labeled with DiI by a modification of the method described by Reynolds and St. Clair (8). A stock solution of the fluorescent probe DiI (Molecular Probes, Inc., Eugene, OR) was prepared by dissolving 30 mg DiI in 1 ml of dimethyl sulfoxide (DMSO), and the latter stock was added to the LDL solution to yield a final ratio of 300 μg DiI to 1 mg LDL protein. After incubating this mixture for 18 h at 37°C, the labeled LDL was reisolated by ultracentrifugation, dialyzed against normal saline, and filter-sterilized as described above.

Commercially available DiI-LDL, which is primarily intended for descriptive morphological studies, and ^{125}I-labeled LDL prepared for quantitative studies of LDL receptor activity were obtained from Biomedical Technologies, Inc. (BTI, Stoughton, MA). These ligands were used for sensitivity comparison studies with DiI-LDL prepared in our laboratory.

Cell culture

Human hepatoblastoma cells, HepG2, obtained from American Type Culture Collection (Rockville, MD) were used to validate our method for quantitating LDL receptor activity by DiI-labeled LDL. Cells were seeded into either 12-well or 24-well plates (Corning, NY), and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, and 0.1 mM nonessential amino acids (Gibco, Grand Island, NY). Cells were grown for 72 h at 37°C in an atmosphere of 5% CO_2 in a humidified incubator and were used for LDL receptor activity determination at a density of 2 million cells/cm^2.

DiI and DiI-LDL standard curve

Standard solutions of DiI were prepared in either chloroform or isopropanol with a concentration range of 10 to 100 ng/ml. Fluorescence was determined in a Perkin-Elmer Model LS-5 spectrofluorometer with excitation and emission wavelengths set at 520 and 578 nm, respectively.

Standard solutions of either our own or the commercially available DiI-LDL were prepared in saline with a concentration range of 100 to 1600 ng protein/ml. One ml of each standard solution was extracted with 1 ml chloroform by vigorous shaking for 1 min, and fluorescence of the chloroform layer was determined as described above. The specific activity of DiI-LDL was then calculated as the amount of DiI (ng) incorporated into 1 μg of LDL protein.

Quantitation of LDL receptor activity in cells

After a period of 72 h culture in DMEM supplemented with FBS, cells were switched to DMEM containing 0.5% human serum albumin (HSA) for an additional 24 h. To determine the dose-response of bound or cell-associated LDL, DiI-LDL (5–200 μg protein/ml) was incubated with HepG2 cells for 2 h at 4°C and 37°C, respectively. At end of the incubation period, cells were extensively washed with phosphate-buffered saline (PBS), 1 ml of isopropanol was added to each well, and plates were gently shaken on a Model 361 orbital shaker (Fisher, Piscataway, NJ) for 15 min. The isopropanol extract of DiI was then transferred to a 10 × 75 mm glass tube, centrifuged at 3000 rpm for 15 min and fluorescence was determined as described above. Cells were dissolved in 2 N NaOH for protein determination.

Studies with ^{125}I-labeled LDL (2.5–200 μg protein/ml) were simultaneously conducted under identical conditions as those described for DiI-LDL, except that after cell washing, 1 ml of 2 N NaOH was added to each well and, after cell lysis was completed (1 h), aliquots were taken for radioactivity and protein determinations. The dose-response of internalized LDL, for either DiI or ^{125}I-labeled, was calculated as the difference between cell-associated (37°C) and membrane-bound (4°C) LDL.

In separate experiments, specific cell-associated LDL was determined as the difference between total (no unlabeled LDL) and nonspecific (plus 500 μg/ml of unlabeled LDL) cell-associated LDL.

Ligand displacement studies were carried out with DiI-LDL or ^{125}I-labeled LDL at a fixed concentration of labeled ligand, 30 μg/ml, and over a concentration range of unlabeled LDL of 0 to 300 μg/ml. After 24-h derepression period, cells were switched to DMEM containing either

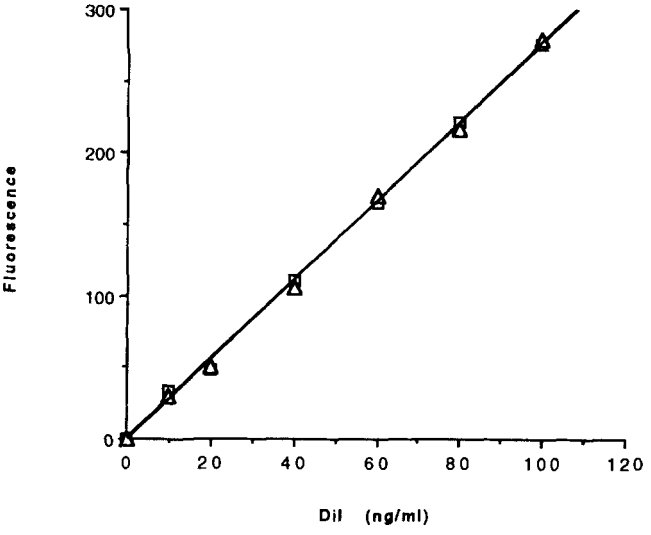

Fig. 1. Standard curve of pure DiI dissolved in either isopropanol (□) or chloroform (△).

one of the two labeled ligands plus increasing concentrations of unlabeled LDL and were incubated at 37°C for 2 h. Subsequently, cells were processed as previously described.

To determine the effect of 1 μM lovastatin (Merck, Rahway, NJ) and 30 μM 25OH-cholesterol (Sigma, St. Louis, MO) on LDL receptor activity, cells were treated with these compounds for 18 h. Subsequently, the media was removed and cells were incubated with 30 μg/ml DiI-LDL at 37°C for 2 h and processed as mentioned above.

For comparison studies of our own DiI-LDL to the commercially available ^{125}I-labeled LDL, cells were treated with the same compounds mentioned above, then exposed to 30 μg/ml of ^{125}I-labeled LDL for 2 h at 37°C. Cells were washed as above, dissolved in 2 N NaOH for determination of radioactivity and cell protein.

RESULTS

Standard curve of DiI, DiI-LDL and labeling efficiency of LDL

The data presented in **Fig. 1** show that, regardless of the solvent used to dissolve DiI, both isopropanol and chloroform gave a linear correlation between DiI concentration and fluorescence with minimum detection limit of 5 ng/ml. This correlation remained linear at least up to a concentration of 100 ng/ml.

Upon extraction of either our own or the commercial (BTI) DiI-LDL with chloroform, the correlation between LDL concentration and fluorescence (**Fig. 2**) was linear over a wide range of LDL protein (100–1600 ng/ml). It is noteworthy that the minimum detection limits of our DiI-LDL and that of BTI were 100 and 500 ng/ml, respec-

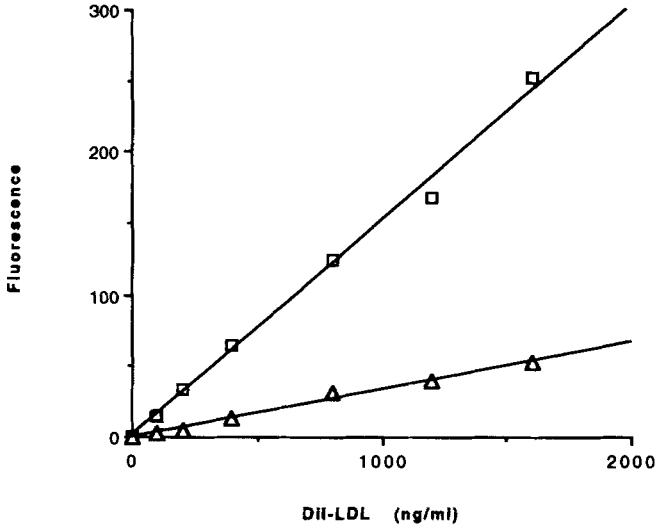

Fig. 2. Concentration-dependent fluorescence of DiI-LDL prepared in our laboratory (□) or obtained from a commercial source (△).

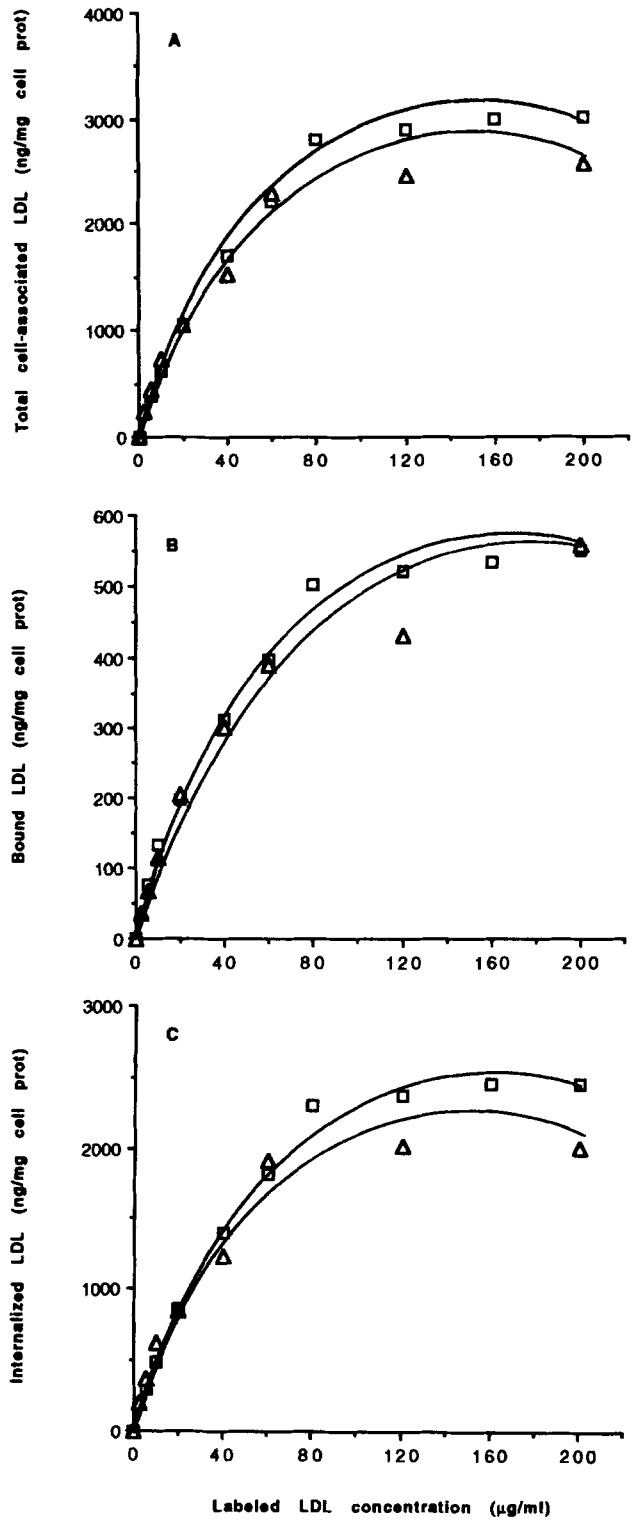

Fig. 3. Dose-response measurement of cell-associated (A), bound (B), and internalized (C) DiI- (□) and ^{125}I-labeled (△) LDL in HepG2 cells. Cells were cultured in 24-well plates and incubated with increasing concentrations of labeled LDL (5–200 μg/ml) for 2 h at either 4°C (to measure binding) or 37°C (to measure cell-association). Internalized LDL was calculated as the difference between (A) and (B). Values are expressed as ng of LDL/mg of cell protein. Each point represents the mean of four wells which varied by less than 10%.

tively. Based on these curves, it is possible to derive the labeling efficiency of DiI-LDL generated commercially and in our laboratory, which were 11 and 56 ng of DiI/μg of LDL protein, respectively. During studies described in this paper, DiI-LDL was prepared on three different occasions and the specific activity obtained varied by less than 10% (52-60 ng DiI/μg LDL protein).

LDL receptor activity with DiI-LDL

Dose-response studies of cell-associated (**Fig. 3A**), bound (Fig. 3B), and internalized (Fig. 3C) DiI-LDL showed a linear, high-affinity response at a concentration range of 5 to 80 μg/ml and appeared to reach a plateau by 200 μg/ml. Except for a slight but statistically insignificant increase in the saturation levels of DiI-LDL over those of ^{125}I-labeled LDL, both ligands displayed essentially identical high-affinity dose-response with respect to cell association, binding and internalization.

Similar dose-response studies were carried out in the linear LDL concentration range of 10 to 80 μg/ml with and without 500 μg/ml unlabeled LDL in order to separate nonspecific from total cell-associated LDL (**Fig. 4**). The data show that a considerable component of total cell-associated LDL was nonspecific and linear up to the highest concentration tested of 80 μg/ml, while the specific component was saturable within a much narrower LDL concentration range of 10 to 40 μg/ml.

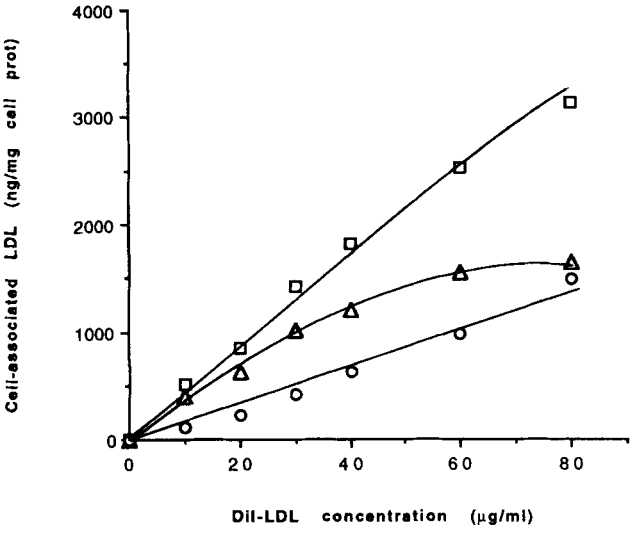

Fig. 4. Dose-response measurement of total (□), specific (△), and nonspecific (○) cell-associated DiI-LDL in HepG2 cells. Cells were cultured in 12-well plates and incubated for 2 h at 37°C in the presence or absence of 500 μg/ml unlabeled LDL. Specific cell-associated LDL was calculated as the difference between total and nonspecific (500 μg/ml LDL) cell-associated LDL. Values are expressed as ng of LDL/mg of cell protein. Each point represents the mean of three wells which varied by less than 10%.

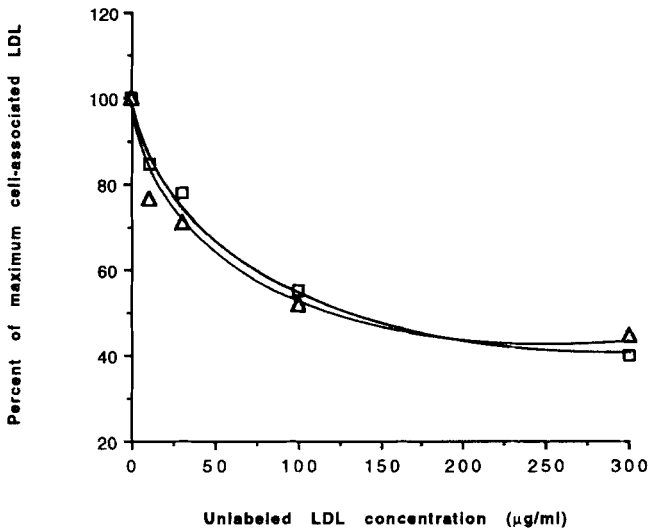

Fig. 5. Competition of either DiI-LDL (□) or ^{125}I-labeled LDL (△) with unlabeled LDL. Cells were cultured in 12-well plates, and competition studies were carried out at 37°C for 2 h. Labeled LDL was used at a constant concentration of 30 μg/ml in the presence of increasing concentrations of unlabeled LDL (0-300 μg/ml). Values are expressed as percent of maximum cell-associated LDL which were 686 ± 36 and 601 ± 38 ng of LDL/mg of cell protein for DiI-LDL and ^{125}I-labeled LDL, respectively. Each point represents the mean of three wells.

Comparison between DiI-LDL and ^{125}I-labeled LDL

The affinity of DiI-LDL to the LDL receptor was compared to that of ^{125}I-labeled LDL in competition studies with native unlabeled LDL. The results shown in **Fig. 5** clearly indicate that DiI-LDL was equally effective as ^{125}I-labeled LDL in displacing unlabeled LDL with half-maximal inhibition (IC_{50}) estimated at 130 and 119 μg/ml, respectively.

The effects of lovastatin (1 μM) and 25OH-cholesterol (30 μM) on LDL receptor activity were examined by using either DiI-LDL or ^{125}I-labeled LDL. The results shown in **Table 1** indicate that first, in control cells, the absolute mass of cell -associated LDL was similar for DiI

TABLE 1. Comparison of DiI-LDL and ^{125}I-labeled LDL in detecting the up-regulation and down-regulation of LDL receptor activity in HepG2 cells

Condition	DiI-LDL	^{125}I-Labeled LDL
	ng/mg cell protein	
Control	655 ± 34	691 ± 25
Lovastatin (1 μM)	866 ± 38[a] (+ 32%)	869 ± 56[a] (+ 26%)
25OH-Chol (30 μM)	255 ± 19[a] (- 62%)	313 ± 15[a] (- 55%)

Values are mean ± SEM; n = 3. Values in parentheses represent % change from control.
[a]Significantly different from control by Student's *t*-test ($P < 0.05$).

and ^{125}I-labeled ligands (655 and 691 ng/mg cell protein, respectively). Second, the up-regulation of LDL receptor activity by lovastatin detected with DiI-LDL (+ 32%) was not different than that detected with ^{125}I-labeled LDL (+ 26%). Finally, 25OH-cholesterol caused a similar degree of suppression of LDL receptor activity whether it was detected by DiI-LDL (−62%) or ^{125}I-labeled LDL (−55%).

DISCUSSION

In this paper we report on the development of a rapid, simple, and nonradioactive method for the quantitative determination of LDL receptor activity using the fluorescent probe, DiI, and its potential application to a high through-put assay of compounds that modulate the LDL receptor in cells. This was possible due to an important modification introduced in the re-extraction of DiI from cells for fluorescence determination and, to a lesser extent, due to highly efficient labeling of LDL with DiI without changing its affinity and interaction with cells.

First, our ability to quantitatively extract DiI from cells and determine its fluorescence was made possible by the use of isopropanol, a plastic-compatible solvent that can be used directly on tissue culture plates (simple and rapid) and has a linear solubilization capacity (Fig. 1) that far exceeds the mass of DiI expected to be associated with cells. It is noteworthy that, in these studies, chloroform was solely used to extract DiI from standard solutions of DiI-LDL in order to determine its specific activity.

Second, it is clear from the data (Fig. 2) that the labeling efficiency obtained with our method (56 ng DiI/μg protein) was at least 5 times greater than that obtained commercially (11 ng DiI/μg protein) and as described by other investigators (4, 19, 20). This improvement of the specific activity was achieved *1)* by increasing the ratio of DiI added to LDL prior to incubation, and *2)* by excluding the d > 1.21 g/ml fraction from the incubation mixture, as this fraction probably diverts a significant amount of DiI from incorporation into the LDL particle. The increased labeling efficiency did not result in any modification in the affinity of LDL to its receptor as demonstrated by the dose-response studies on binding (Fig. 3B), internalization (Fig. 3C), and cell-association (Fig. 3A), both specific and nonspecific (Fig. 4), which tend to agree with data previously generated by our laboratory (21) as well as by other investigators (22, 23) using ^{125}I-labeled LDL as ligand. This was further supported by the results of competition studies (Fig. 5) where DiI-LDL prepared in our laboratory was as effective as ^{125}I-labeled LDL in displacing native unlabeled LDL.

Finally, the DiI-LDL method was successfully adapted to a cell screen using either 12-well or 24-well culture plates where a known up-regulator (lovastatin) and suppressor (25OH-cholesterol) of LDL receptor were tested and resulted in what has been normally obtained when using ^{125}I-labeled LDL as ligand (24). These results provide evidence that the use of DiI-LDL, labeled and later extracted from cells by the method described above, could be easily adapted to a large volume, rapid, and nonradioactive assay of LDL receptor modulators in a cell model. However, despite its ease and versatility, this method cannot quantitate the degradation products of LDL due to the metabolically nonreleasable nature of DiI, and information concerning this catabolic pathway would have to be obtained solely by the ^{125}I-labeled LDL method.

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REFERENCES

1. Kannel, W. B., T. Gordon, and W. P. Castelli. 1981. Role of lipids and lipoprotein fractions in assessing atherogenesis. The Framingham Study. *Prog. Lipid Res.* **20:** 339–348.
2. Bilheimer, D. W., J. L. Goldstein, S. M. Grundy, T. E. Starzl, and M. S. Brown. 1984. Liver-transplantation to provide low-density-lipoprotein receptors and lower plasma-cholesterol in a child with homozygous familial hypercholesterolemia. *N. Engl. J. Med.* **311:** 1658–1664.
3. Lipid Research Clinics Program. 1984. The Lipid Research Clinics Coronary Primary Prevention Trial Results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering. *JAMA.* **251:** 365–374.
4. Pitas, R. E., T. L. Innerarity, J. N. Weinstein, and R. W. Mahley. 1981. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. *Arteriosclerosis.* **1:** 177–185.
5. Barak, L. S., and W. W. Webb. 1981. Fluorescent low density lipoprotein for observation of dynamics of individual receptor complexes on cultured human fibroblasts. *J. Cell Biol.* **90:** 595–604.
6. Rayner, H. C., T. Horsburgh, S. L. Brown, F. L. Lavender, A. F. Winder, and J. Walls. 1990. Receptor-mediated endocytosis of low-density lipoprotein by cultured human glomerular cells. *Nephron.* **55:** 292–299.
7. Reynolds, G. D. 1985. Metabolism of lipoproteins labeled with fluorescent indocarbocyanine dyes for in vitro studies of atherogenesis. *Surv. Synth. Pathol. Res.* **4:** 389–400.
8. Reynolds, G. D., and R. W. St. Clair. 1985. A comparative microscopic and biochemical study of the uptake of fluorescent and ^{125}I-labeled lipoproteins by skin fibroblasts, smooth muscle cells, and peritoneal macrophages in culture. *Am. J. Pathol.* **121:** 200–211.
9. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
10. Goldstein, J. L., S. K. Basu, G. Y. Brunshede, and M. S. Brown. 1976. Release of low-density lipoprotein from its cell-surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.

11. Preobrazhensky, S. N., V. O. Ivanov, I. V. Fuki, V. P. Tsibulsky, A. M. Kameneva, V. S. Repin, and G. A. Ermolin. 1985. Enzyme-linked immunoreceptor assay of low-density -lipoprotein receptors. *Anal. Biochem.* **149:** 269–274.
12. Beiseigel, U., W. J. Schneider, M. S. Brown, and J. L. Goldstein. 1982. Immunoblot analysis of low density lipoprotein receptors in fibroblasts from subjects with familial hypercholestrolemia. *J. Biol. Chem.* **257:** 13150–13156.
13. Gianturco, S. H., K. Hong, M. R. Steiner, D. T. Taunton, R. L. Jackson, A. M. Gotto, Jr., and L. C. Smith. 1979. A laser spectrofluorimeter for studies of cultured cells on the growth surface. *Anal. Biochem.* **92:** 74–81.
14. Barak, L. D., and W. W. Webb. 1982. Diffusion of low-density lipoprotein-receptor complex on human fibroblasts. *J. Cell Biol.* **95:** 846–852.
15. Roach, P. D., and S-P. Noel. 1987. Biotinylation of low density lipoproteins via free amino groups without loss of receptor binding activity. *J. Lipid Res.* **28:** 1508–1514.
16. Roach, P. D., M. Zollinger, and S-P. Noel. 1987. Detection of the low density lipoprotein (LDL) receptor on nitrocellulose paper with colloidal gold-LDL conjugates. *J. Lipid Res.* **28:** 1515–1521.
17. Hojnacki, J. L., R. J. Nicolosi, G. Hoover, N. Llansa, A. G. Ershow, M. El Lozy, and K. C. Hayes. 1978. Comparison of two ultracentrifugation procedures for separation of nonhuman primate lipoproteins. *Anal. Biochem.* **88:** 485–494.
18. Bradford, M. M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
19. Kreiger, M., Y. K. Ho, and J. R. Flack. 1983. Reconstitution of LDL with lipophylic fluoroscein derivatives: quantitative analysis of the receptor activity of human lymphocytes. *J. Receptor Res.* **3:** 361–375.
20. Herman, B., and D. F. Albertini. 1984. A time-lapse video image intensification analysis of cytoplasmic organelle movements during endosome translocation. *J. Cell Biol.* **98:** 565–576.
21. Stephan, Z. F., S. Lindsey, and K. C. Hayes. 1987. Taurine enhances low density lipoprotein binding internalization and degradation by cultured HepG2 cells. *J. Biol. Chem.* **262:** 6069–6073.
22. Dashti, N., G. Wolfbauer, E. Koren, B. B. Knowles, and P. Alaupovic. 1984. Catabolism of human low-density lipoproteins by human hepatoma-cell line HepG2. *Biochim. Biophys. Acta.* **794:** 373–384.
23. Illingworth, D. R., S. Lindsey, and F. C. Hagemenas. 1984. Regulation of low-density lipoprotein receptors in the human hepatoma cell line HepG2. *Exp. Cell Res.* **155:** 518–526.
24. Cohen, L. H., M. Griffioen, L. Havekes, D. Schouten, V. Van Hinsbergh, and H. J. Kempen. 1984. Effects of compactin, mevalonate and LDL on HMG-CoA reductase activity and LDL receptor activity in the human heptoma cell line HepG2. *Biochem. J.* **222:** 35–39.