

Determination of LDL- and scavenger-receptor activity in adherent and non-adherent cultured cells with a new single-step fluorometric assay

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Abstract

Lipoproteins labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) are widely used to visualize LDL- and scavenger-receptor activity in cultured cells. The purpose of this study was to evaluate a new single-step fluorometric assay with high sensitivity for the quantitative determination of the LDL- or scavenger-receptor activity in adherent and non-adherent cells. We used an aqueous solution of 1 g/l SDS dissolved in 0.1 M NaOH to lyse the cells after incubation with DiI-LDL or DiI-acetylated LDL. This allows for the first time the determination of fluorescence intensity and cell protein in the same sample without prior lipid extraction of the fluorochrome. Fluorescence of the cell lysates was determined in microtiter plates with excitation-emission set at 520 and 580 nm, respectively. This rapid method demonstrates high specificity for determining the LDL- and scavenger-receptor activity in cultured cells (e.g., human skin fibroblasts from patients with and without familial hypercholesterolemia; human U-937 monocyte and murine P388 D1 macrophage cell lines). The validity of our fluorescence assay is demonstrated by comparison of cellular uptake and metabolism of lipoproteins labeled with both, DiI and ¹²⁵I. The rapidity and accuracy of this assay allows its routine application for studying receptor-mediated lipoprotein uptake in various cell types.

Keywords: Low density lipoprotein; DiI-LDL; DiI-acetyl-LDL; Fibroblast; Macrophage; Hypercholesterolemia, familial; Fluorescence; Cholesterol

1. Introduction

In vitro studies on cellular metabolism of lipoproteins and lipoprotein receptor activity are mostly performed using lipoproteins labeled with radioactive iodine as a tracer [1,2]. However, radioactive isotopes can be harmful and require special precautions for personal safety as well as waste disposal, thus making this method considerably time consuming and expensive. There are several non-radioactive approaches using the fluorochrome 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

(DiI) as an alternative label for lipoproteins [3]. DiI is thought to associate with the surface of the lipoprotein in a similar way as phospholipids do [4]. DiI labeled native and modified LDL has been used to visualize LDL- and scavenger-receptor activity by fluorescence microscopy in cultured cells as well as in vivo [3,5,6]. In addition, FACS analysis has been applied for semiquantitative determination of binding and uptake of DiI-LDL by fibroblasts, by isolated monocytes and lymphocytes [7–9]. Multi-step extraction procedures for DiI using organic solvents show only limited reproducibility as well as precision, and have not been practicable in routine use [10,11].

The purpose of this study was to evaluate a new single-step fluorometric assay with high sensitivity for the quantitative determination of the LDL- or scavenger-receptor activity in adherent and non adherent cells. This rapid method avoids the need of organic solvents, it compares well with the classical ¹²⁵I radioactive assay and could serve as an alternative approach to the use of radioactive ligands.

Abbreviations: acLDL, acetylated LDL; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DiI-LDL, DiI labeled LDL; DiI-acLDL, DiI labeled acLDL; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; LDL, low density lipoprotein; LPDS, lipoprotein deficient serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate

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2. Materials and methods

2.1. Lipoprotein isolation and labeling

LDL was prepared from freshly drawn CDP-plasma from normolipidemic human donors according to Havel et al. [12]. Plasma density was adjusted to 1.019 g/ml with a high density NaCl solution. In order to prevent oxidation 0.1 g/l EDTA was present during the isolation procedure. After ultracentrifugation at $150\,000 \times g$ for 24 h at 10°C , the infranatant was collected. The pooled infranatant was adjusted to $d = 1.060$ g/ml, recentrifuged as before, and LDL ($d = 1.019$ – 1.060 g/ml) was collected. Lipoprotein deficient serum (LPDS) was obtained after removal of all lipoproteins at $d = 1.25$ g/ml for 48 h. LDL and LPDS preparations were extensively dialyzed against 5 mM Tris, 154 mM NaCl, 0.1 g/l EDTA (pH 7.4) at 4°C .

LDL was labeled with the fluorescent probe 1,1'-dioc-tadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) as described previously [13] with minor modifications. Briefly, LDL was diluted with PBS containing 0.1 g/l EDTA to 1.5 mg of lipoprotein/ml. Nine ml of LPDS (4 g protein/dl) were added to 3 ml of the diluted lipoprotein and mixed with 12 μl of a 100 mM solution of ascorbic acid in order to prevent oxidation. After filtration (0.45 μm , Millipore, Bedford, USA) of the mixture, 225 μl of DiI in DMSO (6 mg/ml) were slowly added under gentle agitation. The mixture was incubated at 37°C for 8 h under nitrogen and light protection. The DiI-labeled LDL was reisolated by ultracentrifugation at $d = 1.060$ g/ml and extensively dialyzed against 5 mM Tris, 154 mM NaCl, 0.1 g/l EDTA, pH 7.4 at 4°C . LDL and DiI-LDL were acetylated, by the method of Fraenkel-Conrat [14] modified for lipoproteins by Basu et al. [15]. All lipoprotein preparations were stored at 4°C in sterile containers after filtration sterilization (0.45 μm). Protein concentrations were determined by the method of Lowry et al. using bovine serum albumin as a standard [16]. Total cholesterol was determined using an enzymatic procedure (Boehringer Mannheim, Germany).

2.2. Cell cultures

Human skin biopsies were obtained from four normo-cholesterolemic donors and eleven patients with familial hypercholesterolemia (FH) with informed consent. Nine subjects were FH heterozygotes, and two FH homozygotes. Fibroblasts derived from skin biopsies were grown in DMEM, containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) [1,17]. P388 D1 (IL-1) (Monocyte-macrophage, mouse; ATCC TIB61) and U-937 (histiocytic lymphoma, human; ATCC CRL 1593) cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640, containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). All cells were maintained in a

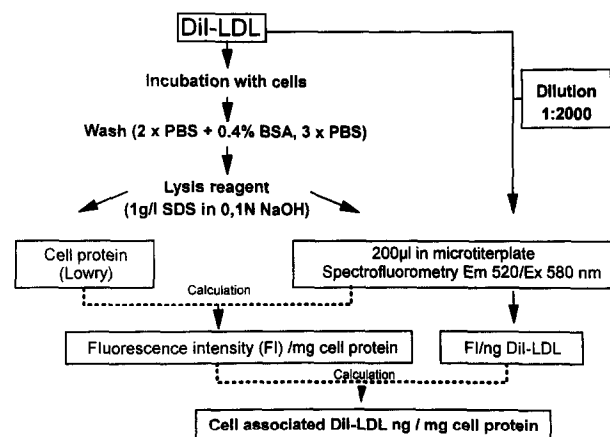


Fig. 1. Flow scheme of the spectrofluorometric assay to determine uptake of DiI-LDL by cultured cells.

humidified incubator at 37°C . In a typical experiment, cell viability exceeded 90% as determined by trypan blue exclusion.

2.3. Quantitative spectrofluorometry of DiI-lipoprotein uptake

Human skin fibroblasts between passages 5–10 were seeded into 35-mm plastic dishes and maintained in DMEM containing 10% FBS, 100 U penicillin/ml and 100 μg streptomycin/ml. After 72 h in culture, the medium was changed to DMEM containing 10% LPDS and the cells were incubated for another 48 h. To study the uptake of DiI-LDL, fibroblasts were incubated for 5 h at 37°C with increasing concentrations of DiI-LDL (5, 10, 20, 50, 100 μg protein/ml). Specificity of uptake was determined at 10 μg DiI-LDL/ml with a 50-fold excess of unlabeled LDL. All incubations were performed in triplicate. After incubation, the cells were washed twice with PBS containing 0.4% BSA and three times with PBS alone. Thereafter 1 ml of cell lysis reagent (1 g/l SDS dissolved in 0.1 M NaOH) was added. Cells were incubated under gentle shaking for 1 h at room temperature. This reagent allowed the direct determination of fluorescence and cell protein in the same sample of lysed cells (Fig. 1). Fluorescence was measured in duplicates of 200 μl of the lysate on black microtiter plates in a Fluorolite 1000 microplate reader (Dynatech Laboratories, Chantilly, USA) with excitation and emission wavelengths set at 520 and 580 nm, respectively. Fluorescence was corrected for the autofluorescence of the lysis reagent, which was only about 10 units on the fluorometer used. Cellular protein was determined in duplicate samples of 40 μl by the method of Lowry, modified for microtiter plates using BSA dissolved in lysis reagent as a standard. In parallel, fluorescence of the DiI-labeled lipoprotein diluted in the lysis reagent (1:2000) was measured in order to determine the specific fluorescence intensity of the DiI-LDL preparation used. Serial

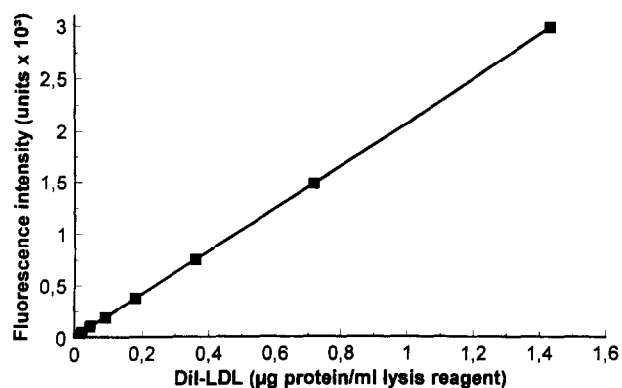


Fig. 2. Fluorescence of DiI-LDL dissolved in lysis reagent. The fluorescence intensity was linear ($r^2 = 0.999$) over a range of 0.005 to 14 μg LDL-protein/ml. The incorporation of DiI into LDL was 27 ng DiI/ μg LDL-protein.

dilution of DiI-LDL in the lysis reagent showed an excellent linear response of fluorescence intensity throughout the range of the fluorometer. In a typical preparation of DiI-LDL (containing 20–30 ng DiI/ μg LDL protein) the minimum detection limit was about

0.005 μg DiI-LDL protein/ml (Fig. 2). Similar results were obtained using dilutions of DiI-acLDL. The recovery of the fluorochrome from DiI-labeled lipoproteins using the aqueous lysis reagent was 100% compared with chloroform as extraction reagent. The extraction of the fluorochrome with other organic solvents, e.g., isopropanol, was incomplete (only 70% recovery compared with chloroform or the lysis reagent).

For experiments with non-adherent cells, U-937 cells were seeded in 35-mm plastic dishes ($3 \cdot 10^6$ /dish). The cells were incubated with increasing concentrations of DiI-LDL (0, 5, 10, 20, 50, 100 $\mu\text{g}/\text{ml}$) at 37°C. Specificity of uptake was determined at 10 μg DiI-LDL/ml with a 50-fold excess of unlabeled LDL. All incubations were performed in RPMI 1640 supplemented with 100 U penicillin/ml and 100 μg streptomycin/ml. The final volume was adjusted to 2 ml/dish. After 5 h of incubation, the cells were transferred into polypropylene centrifuge tubes and washed at 37°C $2 \times$ with PBS containing 0.4% BSA and 0.25 g/l EDTA and $2 \times$ with PBS/EDTA alone. After each wash, cells were centrifuged at $690 \times g$ for 5 min and resuspended in 5 ml of the buffer. The centrifuge tubes were changed before the last wash to reduce contamination with free DiI-LDL adhering to the plastic. After the last centrifugation ($2750 \times g$ for 5 min), the buffer was removed and 1 ml of cell lysis reagent was added to the cell pellet. The further procedure was exactly as described for fibroblasts above.

All fluorometric data have been previously corrected for the autofluorescence of the cells incubated with medium alone. The results were expressed as ng cell associated DiI-LDL per mg of cell protein. Scatchard plot analysis was used for the calculation of K_m and maximal capacity of DiI-LDL uptake [18].

2.4. Studies with lipoproteins labeled with DiI and ^{125}I iodine

DiI-LDL and DiI-acLDL were labeled with ^{125}I as previously described [1,19]. Cells were incubated with [^{125}I]DiI-LDL or [^{125}I]DiI-acLDL as indicated in the legends to figures. At the end of the incubation, the medium was collected and the cells were washed and lysed as described above. Fluorescence, radioactivity, and cell protein were determined in the same sample of the cell lysate. Fluorescence was used to calculate DiI-lipoprotein uptake. The radioactivity in the cell lysate was taken as a measure for cell-associated lipoproteins. Non iodide trichloroacetic acid (TCA)-soluble radioactivity of the medium supernatant served as a measure for lipoprotein degradation. Briefly, 800 μl of the cell supernatant was mixed with 400 μl ice-cold TCA (20% w/v), shaken vigorously for 10 min and centrifuged for 10 min at 4000 rpm. 800 μl of the TCA-supernatant were mixed with 400 μl AgNO_3 (5% w/v), shaken for 10 min and centrifuged for 10 min at 4000 rpm [20,21]. Radioactivity in 1 ml of supernatant was counted.

2.5. Materials

Fatty acid-free bovine serum albumin (BSA) was obtained from Behring (Marburg, Germany).

DMEM with 1000 mg glucose/l and RPMI 1640 were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum was obtained from Biochrom (Berlin, Germany). Sodium ^{125}I iodide was obtained from Amersham-Buchler (Braunschweig, Germany), DiI was obtained from Molecular Probes (Eugene, OR, USA). Sterile culture plastic material was purchased from Nunc (Wiesbaden, Germany).

3. Results

3.1. Suitability of the fluorometric assay for determination of LDL-receptor activity in adherent and non-adherent cells

The concentration-dependent uptake of DiI-LDL (5–100 μg protein/ml) by normal human skin fibroblasts displayed high affinity and saturability (Fig. 3a). The uptake appeared to reach saturation at 30 μg DiI-LDL protein/ml. Scatchard plot analysis of the fluorometric data allowed the calculation of K_m and maximal capacity of DiI-LDL uptake. For normal human fibroblasts the apparent K_m was about 12 μg DiI-LDL protein/ml medium, and the maximal capacity of DiI-LDL uptake was about 10 μg DiI-LDL protein/mg cell protein/5 h. In addition, we measured the uptake of DiI-LDL in a non-adherent human monocyte cell line (U-937), which also express high LDL-receptor activity. The DiI-LDL uptake was receptor-specific and concentration-dependent (Fig. 3b).

3.2. Binding, uptake and metabolism of [125 I]DiI-LDL by human skin fibroblasts

In order to determine whether the uptake of LDL measured as cell-associated fluorescence intensity was comparable to the metabolic processing of [125 I]LDL, we incubated normal human skin fibroblasts at 37°C with increasing concentrations of LDL labeled with both DiI and 125 Iodine. The use of the doubly labeled LDL allowed a direct comparison of both methods in the same dish, thus eliminating factors such as possible differences in the affinity of DiI and 125 I-labeled lipoproteins. Consistent with the results in Fig. 3a, the uptake of [125 I]DiI-LDL displayed the typical saturation kinetics, measured as DiI-fluorescence or the sum of internalized and degraded [125 I]DiI-LDL. As shown in Fig. 4a, the sum of internaliza-

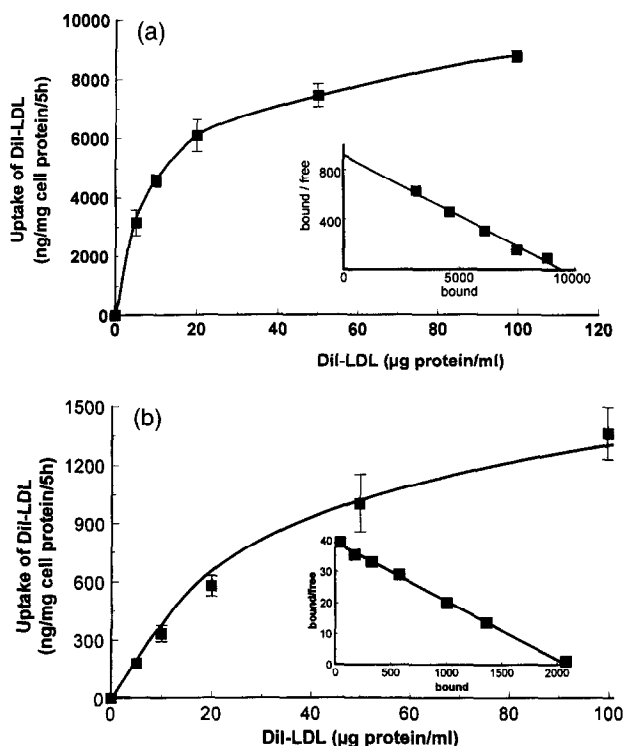


Fig. 3. (a) Concentration-dependent uptake of DiI-LDL by normal human skin fibroblasts. Cells were incubated with 0–100 μ g DiI-LDL protein/ml for 5 h at 37°C. Cells were lysed in 1000 μ l lysis reagent. Values are corrected for nonspecific uptake and represent the mean \pm SD of triplicate dishes. The level of nonspecific uptake was 7%. Cellular protein averaged 0.16 mg/dish. Inset: Scatchard plot analysis of the data indicate a K_m about 10.2 μ g/ml and maximal capacity of uptake of 9.4 μ g/mg cell protein/5 h. (b) Concentration-dependent uptake of DiI-LDL by U 937 cells. The cell suspensions ($3 \cdot 10^6$ cells/ml) were incubated with 0–100 μ g DiI-LDL-protein/ml for 5 h at 37°C. After washing the cells were dissolved in 1 ml of lysis reagent. Values are corrected for nonspecific uptake and represent the mean of triplicate dishes (\pm SD). The level of non-specific uptake was 32%. Cellular protein averaged 0.25 μ g/dish. Inset: Scatchard plot analysis of the data indicate a K_m of about 53 μ g/ml and maximal capacity of uptake of 2.1 μ g/mg cell protein per 5 h.

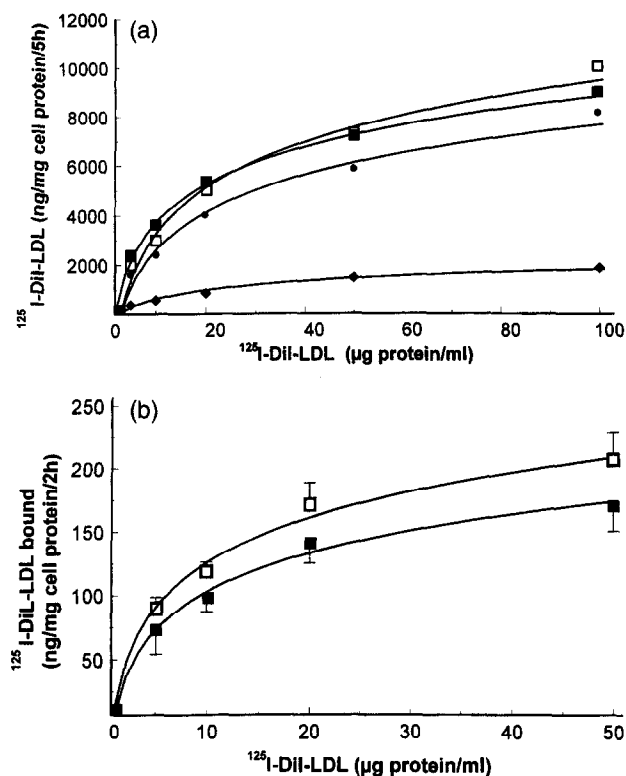


Fig. 4. (a) Concentration-dependent uptake and metabolism of [125 I]DiI-LDL by human skin fibroblasts; comparison of data obtained by determination of DiI-fluorescence and 125 I-radioactivity. Cells were incubated with [125 I]DiI-LDL (0–100 μ g/ml) for 5 h at 37°C. They were analyzed for either DiI-fluorescence intensity (open squares), cell-associated 125 I-radioactivity (closed rhombi) and 125 I-degradation products (closed circles). Total [125 I]LDL metabolized (closed squares) is the sum of cell-associated 125 I-radioactivity and 125 I-degradation products. Values are corrected for nonspecific uptake and degradation and represent the mean of triplicates. Nonspecific degradation was 24% for [125 I]LDL, nonspecific uptake was 13% for [125 I]LDL and 8% for DiI-LDL. The cellular protein averaged 0.10 mg/dish. (b) Comparison of concentration-dependent binding of [125 I]DiI-LDL by human skin fibroblasts. The incubation of 0–50 μ g/ml [125 I]DiI-LDL was performed at 4°C for 2 h. Open squares represent the 125 I-radioactivity and the closed squares represent the DiI-fluorescence intensity of doubly labeled bound LDL. The results were corrected for nonspecific binding and represent the mean of triplicates (\pm SD). The level of non-specific binding was 23% for [125 I]LDL and 10% for DiI-LDL. The cellular protein averaged 0.17 mg/dish.

tion plus degradation of [125 I]DiI-LDL was similar to the data obtained for total cell associated DiI-fluorescence.

Surface binding of doubly labeled [125 I]DiI-LDL was measured at 4°C incubating human skin fibroblasts with increasing concentrations of DiI-LDL (0–50 μ g/ml). The sensitivity of the assay was sufficient enough to measure the amount of cellular surface bound lipoproteins with the use of the DiI fluorescence label. The capacity of [125 I]DiI-LDL binding to normal fibroblasts measured as cell associated fluorescence and radioactivity is shown in Fig. 4b. The radioactivity of the bound [125 I]DiI-LDL was slightly

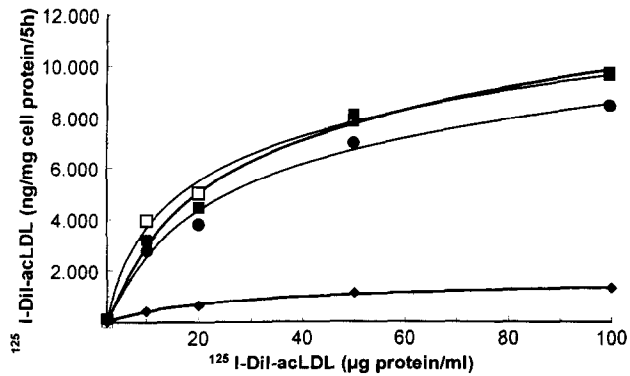


Fig. 5. Concentration-dependent uptake and metabolism of [^{125}I]DiI-acLDL by murine macrophages (P388D1); comparison of data obtained by determination of DiI-fluorescence and [^{125}I]radioactivity. Cells were incubated with [^{125}I]DiI-acLDL (0–100 $\mu\text{g}/\text{ml}$) for 5 h at 37°C. They were analyzed for either DiI-fluorescence intensity (open squares), cell-associated [^{125}I]radioactivity (closed rhombi) and [^{125}I]degradation products (closed circles). Total [^{125}I]acLDL metabolized (closed squares) is the sum of cell-associated [^{125}I]radioactivity and [^{125}I]degradation products. Values are corrected for nonspecific uptake and degradation and represent the mean of triplicates. Nonspecific degradation was 21% for [^{125}I]acLDL, nonspecific uptake was 25% for [^{125}I]acLDL and 17% for DiI-acLDL. The cellular protein averaged 0.33 mg/dish.

but not significantly higher than the bound fluorescence intensity.

3.3. Uptake and metabolism of [^{125}I]DiI-acLDL by murine macrophages

Next we studied the metabolism of doubly labeled [^{125}I]DiI-lipoproteins in murine macrophages (P388D1), in order to determine whether cell-associated fluorescence and radioactivity were also comparable in cells with high scavenger-receptor activity. Macrophages were incubated with increasing concentrations of [^{125}I]DiI-acLDL. As

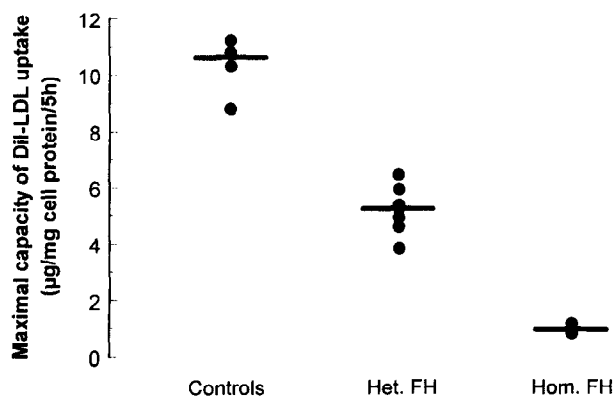


Fig. 6. Maximal capacity of DiI-LDL uptake in cultured fibroblasts from normolipidemic controls and patients with familial hypercholesterolemia. Plasma LDL-cholesterol concentrations (mean \pm S.D.) were 98 ± 36 , 310 ± 43 , 651 ± 69 mg/dl for the controls, FH heterozygotes and homozygotes, respectively. Means of maximal capacity of DiI-LDL uptake of the analyzed fibroblasts are shown by horizontal lines. Data points show the individual values, which represent means of 2 to 3 experiments.

shown in Fig. 5, values obtained by quantitative fluorometry of DiI agreed well with the sum of cellular uptake and degradation of [^{125}I]acLDL.

3.4. Fluorometric determination of LDL-receptor activity in fibroblasts from patients with familial hypercholesterolemia

The suitability of this assay to identify LDL receptor mutations in human fibroblasts from normocholesterolemic and FH subjects was determined using increasing concentrations of DiI-LDL. Scatchard plot analysis of the fluorometric data showed a mean (\pm SD) maximal capacity of DiI-LDL uptake in fibroblasts from normolipidemic subjects, patients with heterozygous FH and homozygous FH of 10.3 ± 1.1 , 5.2 ± 0.8 and 0.9 ± 0.2 $\mu\text{g}/\text{mg}$ cell protein per 5 h (Fig. 6). These results highlight the validity of the fluorometric assay in determining LDL-receptor activity of skin fibroblasts derived from patients with FH.

4. Discussion

The experiments described in this paper show that direct spectrofluorometric determination of DiI after cellular uptake of DiI-labeled lipoproteins and single-step lysis of the cells is a valid technique to quantify LDL-receptor and scavenger-receptor activity in adherent and non-adherent cultured cells. The procedure does not require extraction of the fluorochrome with organic solvents. The validity of this assay has been demonstrated by comparison of cellular uptake and degradation of lipoproteins labeled with both, DiI and [^{125}I] in human fibroblasts, as well as in murine macrophages.

The presently described quantitative fluorometric assay has unique features. First, the fluorescence intensity of DiI from uptake of DiI-labeled lipoproteins can be measured directly in the cell lysate without use of organic solvents and further extraction procedures. This allows a fast spectrofluorometric determination of the DiI-label in microtiter plates with high sensitivity and accuracy. Second, an important advantage of this technique is that the cell protein can be measured in the same sample of the cell lysate also used for the fluorometric determination of DiI. This results in a high accuracy of the data without loss of cell protein, occurring during DiI-extraction from cells with organic solvents. The single-step cell extraction avoids also unrecognized dilution errors during solubilization with sodium hydroxide as it can occur in previously published multi-step extraction procedures [10,11]. Third, the minimal detection limit of specific fluorescence is about 5 ng DiI-LDL-protein/ml under the conditions of our assay, which is more sensitive than that described in previous studies. The extraction of the fluorochrome with the aqueous lysis reagent agrees excellently with the extraction of the dye using

chloroform, whereas the recovery of the fluorochrome using isopropanol [11] is only about 70%. Fourth, the procedure can be easily applied to adherent as well as non adherent cells. Fifth, less oxidation of the LDL is to be expected compared with the labeling procedure with radioactive iodine [22]. Oxidative modification of LDL during the labeling procedure was prevented by the addition of ascorbic acid and EDTA to the incubation mixture. The α -tocopherol content of LDL was not reduced by the labeling procedure (data not shown). Finally, we asked if this technique is as valid and effective as the widely used method using ^{125}I -labeled LDL or acLDL as ligands to study LDL- and scavenger-receptor activity. After uptake of DiI-lipoproteins the fluorochrome is quantitatively retained by the cells [3]. Therefore, the uptake of DiI-lipoproteins by cells should agree with the sum of cell-associated and degraded ^{125}I -labeled lipoproteins [10]. We show that this is true under the conditions of this assay for the uptake and metabolism of doubly labeled [^{125}I]DiI-LDL by human skin fibroblasts as well as for the uptake and metabolism of [^{125}I]DiI-acLDL by murine macrophages. Reynolds and St. Clair found comparable results in monkey smooth muscle cells and monkey skin fibroblasts in separate experiments comparing the cellular uptake of DiI-LDL with total [^{125}I]LDL metabolized [10]. There is also a good correspondence of the K_m value for the LDL receptor in normal human fibroblasts when compared with experiments reported in the relevant literature. In measurements performed with the single-step assay the apparent K_m was $12 \mu\text{g}/\text{ml}$, whereas experiments performed earlier with ^{125}I -labeled LDL resulted in a similar K_m of about $10 \mu\text{g}/\text{ml}$ [1].

The present study shows that DiI-LDL and DiI-acLDL under the conditions of this assay are useful ligands for the quantitative determination of LDL and scavenger-receptor activity in adherent and non-adherent cultured cells. The assay is easy to perform and serves as a valid alternative method to the use of radiolabeled lipoproteins. The rapidity and accuracy of our assay allow its routine application for studying lipoprotein receptor-mediated uptake by various cell types.

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