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note on methodology

A dot-blot assay for the low density lipoprotein receptor

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Summary We describe a new method for detecting the interaction of low density lipoprotein with its receptor using unmodified nitrocellulose as support for membrane protein. The method is specific and sensitive down to 3 μg of membrane protein. Unlabeled LDL, but not HDL, competes with ^{125}I-labeled LDL for binding, and binding is abolished by pretreatment of the membranes with pronase and is dependent upon the presence of Ca^{2+}. Furthermore, modification of arginine or lysine residues on LDL abolishes the lipoprotein interaction with the receptor protein supported on the nitrocellulose. When the membranes are solubilized with octyl glucoside, purification steps of the receptor can be directly followed with no interference of the detergent, therefore eliminating the need for its removal. The increased expression of LDL receptors on liver membranes from estradiol-treated rats was also demonstrated. We suggest, therefore, that this method can be used to detect the presence of LDL receptors on minute amounts of membrane protein. —**Maggi, F. M., and A. L. Catapano.** A dot-blot assay for the low density lipoprotein receptor. *J. Lipid Res.* 1987. **28:** 108–112.

Supplementary key words LDL • detergents • receptor

The low density lipoprotein receptor (LDL-R) is a glycoprotein containing 839 amino acids, with specificity for apolipoproteins B and E (1, 2). This receptor plays a key role in the modulation of the cellular cholesterol homeostasis and plasma cholesterol levels (for reviews see references 3 and 4).

Studies on the expression of the LDL receptor are based upon purification of tissue membrane fractions and binding assays by ultracentrifugation or filtration (5–9). Furthermore, when solubilization of the membranes is required to purify the receptor protein, filter assays are performed after association of the receptor protein to phospholipid vesicles (8). Both methods are difficult to perform when a large number of samples must be processed and only radioactive ligands have been used.

We describe herein a new method for the analysis of the LDL receptor activity on unmodified nitrocellulose using ^{125}I-labeled LDL as a tracer. This assay has the advantage of being easy to perform, does not require treatment of the samples, is specific, and can be quantitated after elution of the silver grains from the X-ray films. Moreover, the assay can be performed on octyl glucoside-solubilized membranes and could be adapted to the use of biotinylated lipoproteins or of apoB or LDL-R antibodies, thus eliminating the need for using radioactive tracers.

MATERIALS AND METHODS

β-Octyl glucoside (OG) and pronase were obtained from Sigma (St. Louis, MO); DEAE-Sepharose was from Pharmacia (Uppsala, Sweden); nitrocellulose was from Bio-Rad (Richmond, CA); and X-ray films were from Kodak (Rochester, NY). All other reagents were analytical grade.

Human LDL was purified by ultracentrifugation (d 1.019–1.063 g/ml) from the plasma of normolipemic donors (10). High density lipoprotein 3 (HDL_3) was separated at d 1.125–1.21 g/ml and further purified by heparin-Sepharose affinity chromatography (11) to remove apoE-containing particles. More than 96% of the LDL protein was apoB as determined by radial immunodiffusion. Neither apoB nor apoE could be detected by SDS gel electrophoresis or radial immunodiffusion in the HDL_3 fraction not retained by the column.

Lipoproteins were labeled with ^{125}I as previously described (12). Free iodine was removed by column chromatography on a Sephadex G-25 column, and further dialyzed against 0.01 M Tris, 0.15 M NaCl, 0.01% EDTA, pH 7.4, for 4 hr at 4°C. The trichloroacetic acid nonprecipitable radioactivity was always less than 1% (range 0.3–0.8%) and the lipid-associated radioactivity was less than 6% (range 4.3–5.9%). Specific activity ranged between 123 and 258 cpm/ng of LDL protein. Lipoproteins were stored at 4°C after sterilization through a 0.22-μm Millipore filter and were used within 7 days from isolation.

Bovine adrenals were obtained from a local slaughterhouse immediately after the animals were killed and were brought to the laboratory in ice within 30 min. Cortexes were separated and the tissue was homogenized with a Polytron tissue homogenizer in buffer A (150 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris, pH 8). Membranes (100,000 *g*) were prepared as described by Kovanen, Brown, and Goldstein (13) at 4°C and stored at −80°C until use.

For the solubilization experiments, membranes were suspended in 125 mM Tris-maleate buffer, pH 6, containing 150 mM NaCl and 2 mM $CaCl_2$ at a final protein con-

Abbreviations: LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor; OG, octyl glucoside.

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centration of 7–9 mg/ml. Octyl glucoside was added to a final concentration of 40 mM. The suspension was sonicated three times for 20 sec with a Branson sonicator at the maximum setting and the undissolved material was pelleted by centrifugation at 100,000 *g* for 1 hr at 4°C.

The OG-soluble material was applied to a DEAE-Sepharose column equilibrated with buffer B (50 mM Tris-maleate containing 2 mM CaCl and 40 mM octyl glucoside, pH 6) after a 1:4 dilution with the same buffer. The column was then eluted with a linear gradient of NaCl from 0 to 300 mM in buffer B at a flow rate of 2 ml/min. Fractions of ca. 2 ml were collected.

The dot-blots were prepared using a manifold apparatus (Bio-Rad). Aliquots of the membranes or of the solubilized proteins were applied to the nitrocellulose in a volume of about 50 μl. The well was washed with 150 μl of buffer B. The nitrocellulose unbound sites were then saturated by incubation for 1 hr at room temperature in 0.01 M Tris, 50 mM NaCl, and 2 mM $CaCl_2$, pH 8, containing 3% bovine serum albumin w/v. The incubation with the labeled LDL was carried out in the same buffer containing 10 μg/ml of ^{125}I-labeled LDL for 1 hr followed by three washes (15 min each) with buffer without LDL at room temperature.

The nitrocellulose was then dried and processed for autoradiography. The silver grains were eluted from the exposed films with 1 N NaOH as described by Suissa (14) and the absorbance at 500 nm was measured.

The effect of unlabeled lipoproteins on the LDL binding was determined in the presence of a 50-fold excess of cold lipoproteins. The interference of heparin was evaluated by the addition to the binding buffer of 4 mg/ml of heparin. The effect of pronase treatment on the binding was determined after incubation of the membrane suspension with the proteolytic enzymes (final concentration,

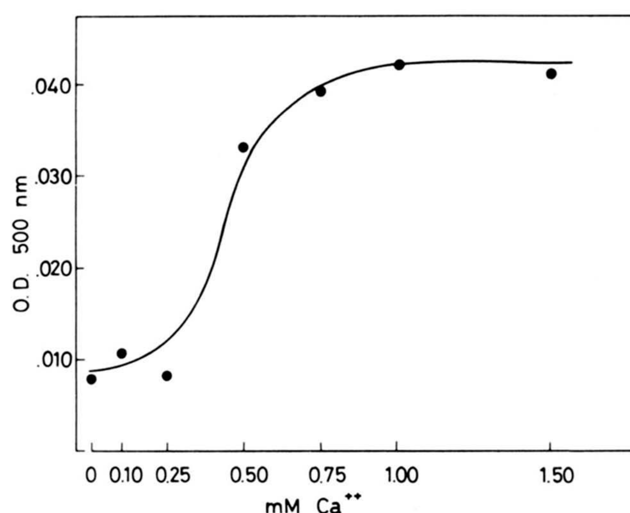

Fig. 2. ^{125}I-Labeled LDL specific binding to adrenal membranes as a function of the Ca^{2+} present in the incubation medium. Each point is the mean of triplicate determinations that did not differ by more than 6%.

5 μg/ml). The reaction was quenched by the addition of bovine serum albumin (3 mg/ml final concentration) and the membranes were directly applied to the nitrocellulose using the dot-blot apparatus. The sensitivity of the binding to the presence of calcium was determined using buffers containing increasing concentrations of Ca^{2+}, while the effect of EDTA was evaluated by the addition of 30 mM disodium EDTA to the incubation medium. The ability of chemically modified LDL to compete with ^{125}I-labeled LDL for the binding in this system was evaluated after modification of the LDL with cyclohexadione or methylation (15, 16).

The saturability of the LDL binding to adrenal membranes was also determined using increasing amounts of

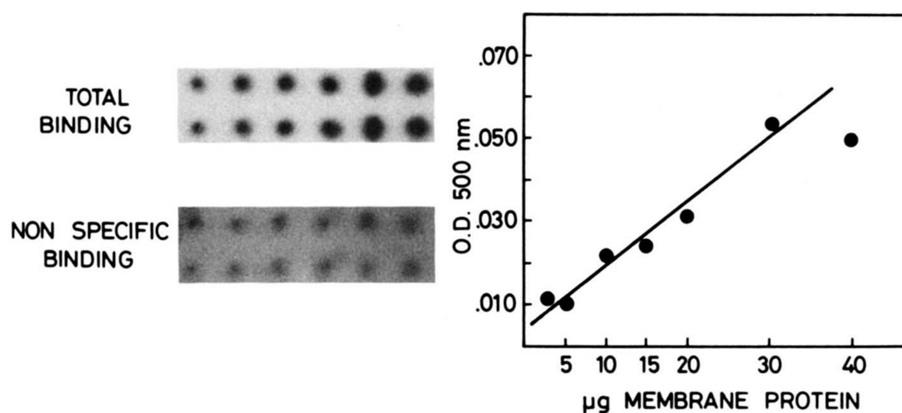

Fig. 1. ^{125}I-Labeled LDL specific binding to adrenal membranes as a function of the concentration of the membrane protein. Each point is the mean of triplicate determinations that did not differ by more than 8%.

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TABLE 1. Effect of heparin, EDTA, and HDL_3 on the binding of ^{125}I-labeled LDL

Experiment	Heparin (4 mg/ml)	EDTA (30 mM)	HDL_3 (500 μg/ml)
	% of specific binding remaining		
1	15.3	15.2	101
2	11.2	8.0	109
3	9.5	ND	98

Experiments were performed as described in Materials and Methods. LDL (10 μg/ml) were incubated for 1 hr in the presence of the indicated amounts of heparin, EDTA, or HDL_3. The nitrocellulose was then dried and processed for autoradiography. The specific binding was the difference between total binding and the nonspecific binding detected as binding in the presence of 500 μg/ml of unlabeled LDL. Data are means of quadruplicate determinations that did not differ by more than 6%. ND, not determined.

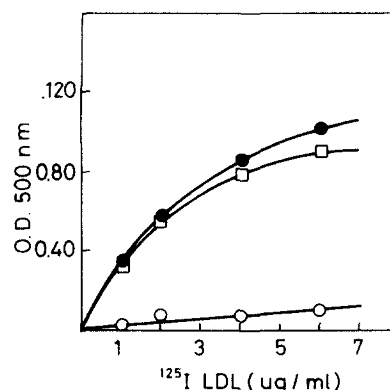

Fig. 3. ^{125}I-Labeled LDL binding to adrenal membranes as a function of the concentration of ^{125}I-labeled LDL. The total (●), specific (□), and nonspecific (○) binding are presented. Each point is the mean of triplicate determinations that did not differ by more than 8%.

LDL. These incubations were performed as described above with a range of LDL concentration from 0.5 to 6 μg of protein/ml.

To show that the dot-blot method can detect differences in the expression of lipoprotein receptor, we used liver membranes prepared from control and estradiol-treated rats (13). Male Sprague-Dawley rats (250–300 g) were treated with 17α-ethinyl estradiol (5 mg/kg per day) subcutaneously for 5 days. Plasma cholesterol levels dropped to 5–10 mg/dl. Liver membranes were prepared as for adrenal cortexes and the assay was performed exactly as described.

RESULTS AND DISCUSSION

The results of a typical assay are reported in **Fig. 1.** Sensitivity of the assay was down to less than 3 μg of membrane protein applied to the nitrocellulose and was linear up to 30 μg of protein (Fig. 1). Binding was complete after 20 min of incubation (data not shown). The deviation from linearity at 40 μg of protein could be due to the capacity of the nitrocellulose (for proteins) that might have been saturated at this concentration of membrane protein. In the attempt to improve the sensitivity of the method in some experiments, we also used ^{125}I-labeled βVLDL from cholesterol-fed rabbits. Results, however, were comparable to those obtained with LDL (data not shown); we therefore chose to use the LDL for further studies.

The specificity of the LDL binding to the cellulose-supported membranes was determined in several ways. Binding was dependent upon Ca^{2+} with a maximum at 0.75 mM Ca^{2+} (**Fig. 2**) and 30 mM EDTA competed with LDL very effectively (**Table 1**). Furthermore excess unlabeled LDL and heparin (4 mg/ml), but not HDL_3, competed for the binding (Table 1). Pronase treatment of the membranes effectively reduced the LDL binding (**Table 2**) thus demonstrating that a protein-LDL interaction is detected. The specificity of the binding was confirmed by the ineffectiveness of chemically modified LDL in com-

TABLE 2. Effect of pronase and chemically modified LDL on the binding of ^{125}I-labeled LDL

Experiment	Pronase (5 μg/ml)	Cyclohexadione-Modified LDL (500 μg/ml)	Methylated LDL (500 μg/ml)
	% of specific binding remaining		
1	1.5	94	ND
2	0	ND	92

Binding was determined as described in Table 1. Data are means of quintuplicate determinations that did not differ by more than 8%. ND, not determined.

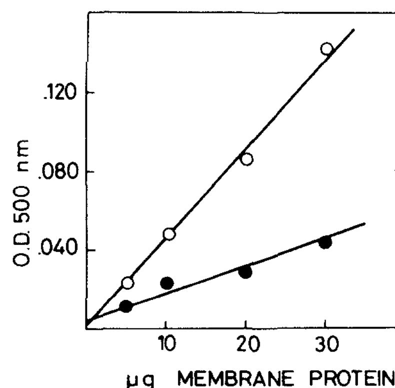

Fig. 4. ^{125}I-Labeled LDL binding to rat liver membranes form control (●) and estradiol-treated (○) animals as a function of membrane protein concentration. Each point is the mean of triplicate determinations that did not differ by more than 10%.

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peting for the binding with labeled LDL (Table 2). All these characteristics are compatible with the detection of a specific LDL-receptor protein interaction. The LDL-receptor interaction in fact is Ca^{2+}-dependent (9); heparin displaces LDL (17) and the binding is pronase-sensitive (9). Modification and arginyl or lysyl residues of LDL abolishes binding (15, 16), and HDL_3 does not compete with LDL for binding to liver membranes (13) as well as to adrenals and fibroblasts (1, 2).

The saturation curve for LDL binding is shown in **Fig. 3.** Binding was saturable at concentrations of about 5 μg/ml of ^{125}I-labeled LDL. The saturability of the binding further emphasizes the concept that the interaction of LDL with a specific receptor is being detected.

The binding of LDL to membranes from estradiol-treated rats dramatically increased as shown in **Fig. 4.** A threefold increase was found, consistent with the results obtained with the ultracentrifuge assay (data not shown). Kovanen et al. (13) reported a three- to eightfold increased binding of ^{125}I-labeled LDL to rat liver membranes after 17α-ethinyl estradiol treatment that induced a dramatic decrease of plasma cholesterol. In our rats after estradiol treatment, plasma cholesterol decreased to 5–10 mg/dl.

To demonstrate that this assay can also be performed on detergent-solubilized proteins, we tested the effect of either Triton X-100 or octyl glucoside on the binding of LDL to the membranes. While octyl glucoside did not interfere with the assay up to a concentration of 60 mM, Triton X-100 gave a very high, nonspecific binding which was also present when other proteins were applied to the nitrocellulose (data not shown). Interference of Triton X-100 with LDL binding has been reported (8); the reasons for this, however, are unknown.

The specificity of the assay was also demonstrated by partial purification of the LDL receptor protein on a DEAE column. The binding activity was all recovered with a retained fraction that eluted at 160–190 mM NaCl (**Fig. 5**). We have also shown that the peak corresponds to the LDL binding activity as determined by the method of Schneider et al. (8) (data not shown) and by the ligand blotting assay (18). An advantage of the proposed method, however, it that it can be applied to detergent-solubilized membranes. Detergent removal is a prerequisite when the eluate of the purification step must be studied for the LDL receptor activity (8). The method reported in this study allows direct application of membranes solubilized with OG with no apparent interference with the binding. We have also successfully applied this method to the binding of ^{125}I-labeled HDL_3 to liver and adrenal membranes (data not shown).

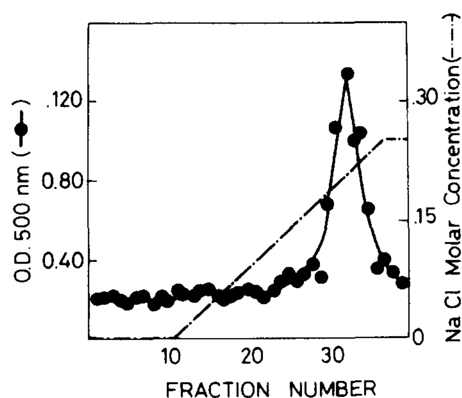

Fig. 5. ^{125}I-Labeled LDL binding to the fractions eluted from a DEAE-Sepharose column, pH 6. Aliquots of the fractions were applied to the nitrocellulose and the binding was performed as described in Materials and Methods. Each point is the mean of duplicate determinations that did not differ by more than 10%.

In summary, we have described a method that allows the detection of LDL receptors on crude membranes as well as on detergent-solubilized membranes. This method is easy to perform and does not require prior removal of the detergent. Furthermore, it can be applied to minute amounts of sample. It also holds the potential for using nonradioactive tracers such as biotinylated ligands or antibodies. Work is in progress along these lines.

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