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Detection of the low-density-lipoprotein receptor with biotin–low-density lipoprotein

A rapid new method for ligand blotting

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A new technique has been developed to identify low-density-lipoprotein (LDL) receptors on nitrocellulose membranes, after transfer from SDS/polyacrylamide gels, by ligand blotting with biotin-modified LDL. Modification with biotin hydrazide of periodate-oxidized lipoprotein sugar residues does not affect the ability of the lipoprotein to bind to the LDL receptor. Bound lipoprotein is detected with high sensitivity by a streptavidin–biotin–peroxidase complex, and thus this method eliminates the need for specific antibodies directed against the ligand. The density of the bands obtained is proportional to the amount of pure LDL receptor protein applied to the SDS/polyacrylamide gel, so that it is possible to quantify LDL receptor protein in cell extracts. Biotin can be attached to other lipoproteins, for example very-low-density lipoproteins with β-mobility, and thus the method will be useful in the identification and isolation of other lipoprotein receptors.

Proteins transferred to nitrocellulose membranes after SDS/polyacrylamide-gel electrophoresis can be detected by immunoblotting with labelled specific antibodies or by ligand blotting with high-affinity ligands (for review, see Towbin & Gordon, 1984). The receptor for plasma LDL (LDL receptor) has been detected in extracts of bovine adrenal glands and of human skin fibroblasts by ligand blotting (Daniel *et al.*, 1983). The native cell-surface LDL receptor exhibits Ca^{2+}-dependent binding of lipoproteins containing apoB or apoE, and these binding characteristics are preserved in the blotted protein if the detergent-solubilized receptor is subjected to SDS/polyacrylamide-gel electrophoresis under non-reducing conditions. LDL bound to the blotted receptor has been detected directly by autoradiography after incubation with ^{125}I-labelled LDL (Kroon *et al.*, 1984) or after a second incubation with ^{125}I-labelled anti-LDL antibodies (Daniel *et al.*, 1983), and by a peroxidase-linked second-antibody system (Dresel *et al.*, 1984). In the present paper we report a new method for the detection of bound ligand based on the high-affinity biotin–streptavidin interaction (Guesdon *et al.*, 1979), which eliminates the need for either radiolabelling or specific antibodies. LDL to which biotin has been covalently bound (biotin–LDL) is incubated with the nitrocellulose membrane containing blotted proteins, and any biotin–LDL bound to proteins is detected with high sensitivity by a streptavidin–biotinylated-peroxidase complex. The modification reaction with biotin is applicable to other lipoprotein ligands, and is sufficiently mild to prevent any loss of biological activity of the lipoprotein. The method should prove useful in detecting different types of lipoprotein-binding protein in a variety of tissue from different species.

Abbreviations used: LDL, low-density lipoprotein(s); HDL, high-density lipoprotein(s); VLDL, very-low-density lipoprotein(s); LPDS, lipoprotein-deficient serum; apoE and apoB, apo-lipoprotein E and -lipoprotein B; FCS, foetal-calf serum.

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Materials and methods

4-Chloro-1-naphthol and biotin hydrazide were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Streptavidin–biotinylated-peroxidase complex was purchased from Amersham International, Amersham, Bucks., U.K. Heparin–Sepharose CL 6B was obtained from Pharmacia, Milton Keynes, Bucks., U.K. All constituents of tissue-culture media were purchased from Gibco

Europe Ltd., Paisley, Renfrewshire, Scotland, U.K.

Isolation of lipoproteins

Human LDL ($\rho = 1.019$–1.063 g/ml) was isolated from the plasma of a patient with familial hypercholesterolaemia. After preliminary centrifugation of the plasma at $\rho = 1.019$ g/ml for 16h at 50000 rev/min at 8°C in a Beckman 70 Ti rotor, the infranatant was adjusted to $\rho = 1.30$ g/ml, layered under 0.15M-NaCl/1mM-EDTA, pH7.4 ($\rho = 1.006$ g/ml) and centrifuged in a Beckman VTi 50 vertical rotor for 2½h at 50000 rev./min at 10°C as described by Chung *et al.* (1980). The visible LDL band was removed with a syringe, adjusted to $\rho = 1.063$ g/ml with KBr and washed by centrifugation at 43000 rev./min for 16h at 8°C in a Beckman SW 50.1 rotor. Human HDL ($\rho = 1.063$–1.21 g/ml) were isolated by essentially the same method. The visible HDL band was removed by syringe, adjusted to $\rho = 1.21$ g/ml with KBr and washed by centrifugation for 30h at 8°C at 50000 rev./min in a Beckman SW 50.1 rotor. LDL and HDL were dialysed against 0.15M-NaCl/1mM-EDTA, pH7.4, passed through a 0.45μm-pore-size Millipore filter and stored sterile at 4°C.

ApoE-free HDL was prepared by passing total HDL through a column of heparin–Sepharose CL 6B as described by Weisgraber & Mahley (1980). Unbound protein was dialysed against phosphate-buffered saline, pH7.4, and a portion was subjected to SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). No apoE was detectable when 50μg of HDL protein was applied to the gel.

Human βVLDL (VLDL with β-mobility) was isolated from the plasma of a patient with Type III hyperlipoproteinaemia by ultracentrifugation at $\rho = 1.006$ g/ml for 16h at 8°C at 50000 rev./min in a Beckman 70 Ti rotor, followed by re-centrifugation of the floating VLDL at the same density in a Beckman SW 50.1 rotor for 16h at 43000 rev./min at 8°C. The βVLDL was passed through a Millipore filter (0.45μm pore size) and stored sterile at 4°C.

Lipoprotein-deficient serum was prepared from the infranatant solution obtained after isolation of LDL from the plasma of normal donors, as described by Knight & Soutar (1982).

^{125}I-labelled LDL was prepared by a modification of the iodine monochloride method (McFarlane, 1958) as described by Bilheimer *et al.* (1975).

Modification of lipoproteins with biotin

Lipoproteins were dialysed for at least 2h against phosphate-buffered saline (137mM-NaCl/2.7mM-KCl/8.1mM-Na_2HPO_4/1.5mM-KH_2PO_4/0.9mM-$CaCl_2$/0.5mM-$MgCl_2$), pH7.4. LDL (8mg of protein/ml) was oxidized by the addition of sufficient 0.1M-sodium metaperiodate to give a final concentration of 4mM. After 30min at 0°C, the protein was freed from sodium metaperiodate by passage through a column (1cm × 10cm) of Sephadex G-25 equilibrated with phosphate-buffered saline, pH7.4. The oxidized LDL (2mg of protein) was incubated with biotin hydrazide (5.0mg) in phosphate-buffered saline in a final volume of 2.5ml. In some experiments the ratio of biotin hydrazide to LDL protein was varied by the addition of different amounts of biotin hydrazide, as described in the Results and discussion section. The conjugation mixture was incubated for 30min at room temperature, then 0.1M-sodium cyanoborohydride was added to give a final concentration of 1mM and the mixture incubated overnight at 4°C. Excess reagents were removed by exhaustive dialysis of the biotin–LDL for at least 24h against several changes of phosphate/buffered saline, pH7.4. The modified lipoprotein was passed through a Millipore filter (0.45μm pore size), stored sterile at 4°C and used within 2 weeks. HDL and βVLDL were modified by the same method, with 2mg of apoprotein and 2mg of biotin hydrazide in the conjugation mixture.

Ligand blotting

LDL-receptor activity in bovine adrenal glands was partially purified by DEAE-cellulose chromatography (DEAE fraction) and further purified by affinity chromatography on LDL–Sepharose 4B as described by Schneider *et al.* (1982). Samples were fractionated by SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose membranes as described by Beisiegel *et al.* (1982). The gels were calibrated with the following standard proteins: myosin (M_r 205000); β-galactosidase (116000); phosphorylase *b* (97400); bovine serum albumin (66000); ovalbumin (43000) (Sigma high-M_r standard mixture; Sigma Chemical Co.). The nitrocellulose strips to which the standard proteins were transferred were stained by immersion for 5min in 0.1% Amido Black B in aq. 45% (v/v) methanol containing acetic acid (10%, v/v), and destained in acetic acid (10%, v/v).

For ligand blotting with biotin–LDL, strips were pre-incubated for 30min at 37°C in 50mM-Tris/HCl, pH8.0, containing 2mM-$CaCl_2$, 90mM-NaCl and 50mg of bovine serum albumin/ml (buffer A) to decrease non-specific binding. All subsequent incubations were carried out at room temperature on a rocking platform (Denley, Bolney, Sussex, U.K.). The strips were incubated for 1h with the biotin-modified lipoprotein (20μg of protein/ml) in buffer A, and then washed once rapidly, twice for 20min each and once more rapidly in washing buffer (50mM-Tris/HCl, pH8.0, containing 2mM-$CaCl_2$, 90mM-NaCl and

5mg of bovine serum albumin/ml). The strips were then incubated for 30min with streptavidin–biotinylated-peroxidase complex in buffer A adjusted to pH7.4 (diluted 300-fold as suggested by the manufacturer) and then washed by the procedure described above in 10mM-Tris/HCl, pH7.4, containing 0.15M-NaCl and 5mg of bovine serum albumin/ml. After a rapid rinse in water, the strips were incubated in substrate solution [4-chloro-1-naphthol (0.4mg/ml), H_2O_2 (0.03%) in 10mM-Tris/HCl, pH7.4 (Dresel *et al.*, 1984)]. After 5–10min, the strips were rinsed in water to stop the reaction, and air-dried on blotting paper in the dark to prevent fading of the colour. Band intensity was compared by densitometric scanning of the strips or of black-and-white photographs of the strips with a Joyce–Loebl Chromoscan 3.

Fibroblast binding experiments

Normal human skin fibroblasts were seeded at a concentration of 5×10^4 cells/dish into 60mm-diameter plastic Petri dishes containing 3ml of Eagle's minimum essential medium with Earle's salts, 20mM-Tricine, 25mM-$NaHCO_3$, penicillin (100 units/ml), streptomycin (100μg/ml) and 10% FCS. Cells were re-fed with this medium on day 2, and on day 5 the cells were washed once with 3ml of Puck's saline A and incubated with the above medium without FCS, containing LPDS at a concentration of 2.5mg of protein/ml. On day 6 this medium was replaced by 2ml of the LPDS-containing medium without $NaHCO_3$, and the cells incubated at 4°C for 1h. ^{125}I-labelled LDL was added to each dish, together with unlabelled lipoproteins at different concentrations, and the cells were incubated at 4°C for 2h. Each dish was then washed rapidly three times, and twice for 10min each, in ice-cold 150mM-NaCl/50mM-Tris/HCl buffer, pH7.4, containing 2mg of bovine serum albumin/ml. After a final wash in this buffer without albumin the cells from each dish were solubilized in 2ml of 0.1M-NaOH, and assayed for ^{125}I radioactivity. Portions were taken for protein determination by the method of Lowry *et al.* (1951). Binding was expressed as ng of LDL bound/mg of cell protein and was the mean value for duplicate dishes.

Results and discussion

The usual method of linking biotin to proteins is by reaction with biotin *N*-hydroxysuccinimide, which results in the formation of covalent bonds between biotin and the free amino side groups of lysine (Guesdon *et al.*, 1979). However, free lysine amino groups are known to be required for the interaction of apoB or apoE with the LDL receptor (Weisgraber *et al.*, 1978); in the present study, therefore, biotin was conjugated to lipoproteins via sialic acid residues on the apoproteins, by allowing periodate-oxidized lipoproteins to react with biotin hydrazide (Gahmberg & Anderson, 1977).

To demonstrate that the modification with biotin hydrazide did not affect the interaction of the lipoprotein with the LDL receptor, biotin–LDL was incubated with human skin fibroblasts in the presence of ^{125}I-labelled unmodified LDL. LDL modified to different extents with biotin was as effective as unmodified unlabelled LDL in competing with ^{125}I-LDL for binding to the LDL receptor at 4°C (Fig. 1). Although no attempt was made in this study to determine whether periodate treatment resulted in any oxidation of lipoprotein lipids, any such changes were without effect on the ability of the lipoproteins to bind to the LDL receptor.

When biotin–LDL was incubated with a nitrocellulose strip to which an electrophoretically separated DEAE-cellulose-purified extract of bovine adrenal glands had been transferred, and the strip then incubated with the streptavidin–biotinyl-

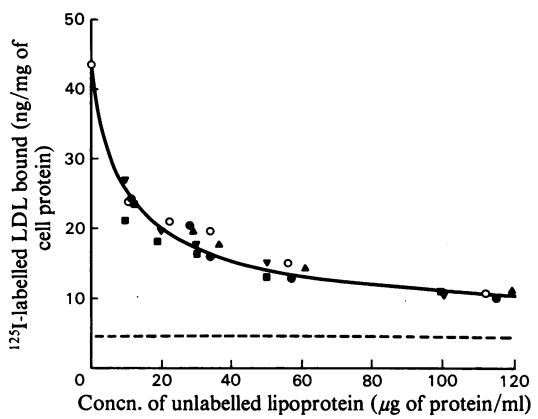

Fig. 1. *Effect of unlabelled biotin–LDL on the binding of ^{125}I-labelled LDL to human skin fibroblasts at 4°C*
Normal human skin fibroblasts were cultured for 5 days in medium containing 10% FCS and then incubated for 24h in medium containing human LPDS (2.5mg of protein/ml). On the day of the experiment the cells were pre-incubated for 1h at 4°C in 2ml of the same medium without bicarbonate. ^{125}I-LDL (sp. radioactivity 59c.p.m./ng of protein) was added to give a final concentration of 4μg of protein/ml together with different concentrations of unlabelled LDL (○) or unlabelled LDL modified with biotin (closed symbols). Biotin–LDL was prepared with different ratios by weight of biotin hydrazide to LDL protein in the incubation mixture as follows: ●, 2.5:1; ■, 1:1; ▼, 0.5:1; ▲, 0.125:1. The broken line indicates the amount of ^{125}I-labelled LDL bound in the presence of an excess of unlabelled unmodified LDL (1.0mg/ml).

ated-peroxidase complex as described in the Materials and methods section, a band was detected on the nitrocellulose strip that corresponded to a protein of M_r approx. 130000 (Fig. 2). This is similar to the M_r of the blotted LDL receptor detected by Daniel *et al.* (1983). A minor band of M_r approx. 97000 was also detectable (Fig. 2, lanes 1 and 4). Since this band is also recognized by a monoclonal antibody to the bovine LDL receptor (A. K. Soutar, D. P. Wade & B. L. Knight, unpublished work), it may represent either a minor proteolytic fragment of the LDL receptor, or a precursor form of the mature receptor protein (Huettinger *et al.*, 1984). Incubations with biotin–LDL were carried out at a lipoprotein protein concentration of 20 μg/ml. Increasing this concentration did not result in any increase in the intensity of the bands obtained (results not shown). Binding of biotin–LDL was inhibited in the presence of either 10mM-EDTA or an excess of unmodified LDL (Fig. 2), a finding in accordance with previously described characteristics of LDL binding to the LDL receptor (Daniel *et al.*, 1983). Binding was specific, since biotin-modified apoE-free HDL showed no detectable binding (Fig. 2). βVLDL is known to bind to the LDL receptor on human skin fibroblasts (Hui *et al.*, 1984). Biotin-modified human βVLDL showed EDTA-sensitive binding to the LDL receptor (Fig. 2), confirming that this method may be used to detect the binding of lipoproteins other than LDL to the nitrocellulose-immobilized LDL receptor.

To determine the conditions for preparing biotin–LDL that gave maximum sensitivity in detecting the LDL receptor on nitrocellulose membranes, biotin–LDL was prepared with various ratios of biotin hydrazide to LDL protein in the reaction mixture, a procedure that should result in LDL modified with different amounts of biotin. Maximum sensitivity was obtained when the ratio of biotin hydrazide to LDL protein was 2.5:1 (w/w). Lower ratios resulted in biotin–LDL preparations that apparently bound less of the streptavidin–biotinylated-peroxidase complex, whereas increasing the ratio to 7.5:1 did not increase the intensity of the bands obtained as determined by densitometric scans of the strips (Fig. 3).

Subsequent experiments were carried out with biotin–LDL prepared with a ratio of biotin hydrazide to LDL protein of 2.5:1. With a highly purified LDL-receptor fraction that gave one major band of approx. M_r 130000 when stained for

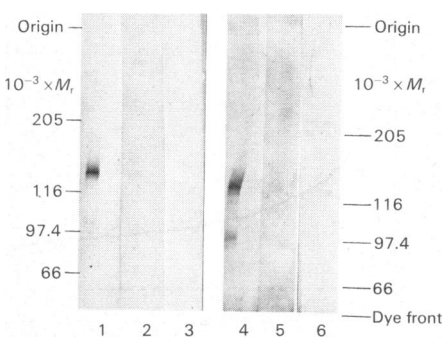

Fig. 2. *Detection of the LDL receptor on nitrocellulose membranes by ligand blotting with biotin–LDL*
Semi-purified LDL receptor from bovine adrenal glands (DEAE–cellulose fraction; total protein/lane = 40 μg) was fractionated by SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose membranes. The strips were incubated with biotin-modified lipoproteins at 20 μg of protein/ml under different conditions: 1, biotin–LDL; 2, biotin–LDL + 10mM-EDTA; 3, biotin/LDL + excess unmodified LDL (1.0mg/ml); 4, biotin–βVLDL; 5, biotin–βVLDL + 10mM-EDTA; 6, biotin–HDL. Bound ligand was detected by incubation of the strips with streptavidin–biotinylated-peroxidase followed by substrate solution (4-chloro-1-naphthol) as described in detail in the Materials and methods section. The M_r of ligand-binding proteins was estimated by comparison of the strip with a parallel strip containing transferred standard proteins that was stained with 0.1% Amido Black.

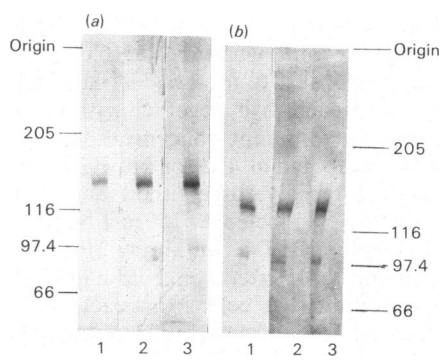

Fig. 3. *Effect of varying the ratio of biotin to LDL protein in the modification reaction on the sensitivity of the detection of the LDL receptor by ligand blotting with biotin–LDL*
Partially purified LDL receptor from bovine adrenal glands DEAE-cellulose fraction [(*a*) 30 μg and (*b*) 40 μg of protein/lane] was fractionated by SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose membranes. Strips were incubated with biotin–LDL prepared with different ratios by weight of biotin hydrazide to LDL protein in the reaction mixture. The results shown are from two separate experiments: (*a*) 1, biotin/LDL protein, 0.125:1; 2, 0.5:1; 3, 2.5:1; (*b*) 1, 2.5:1; 2, 5.0:1; 3, 7.5:1. The M_r of ligand-binding proteins was estimated by comparison of the strip with a parallel strip containing transferred standard proteins that was stained with 0.1% Amido Black.

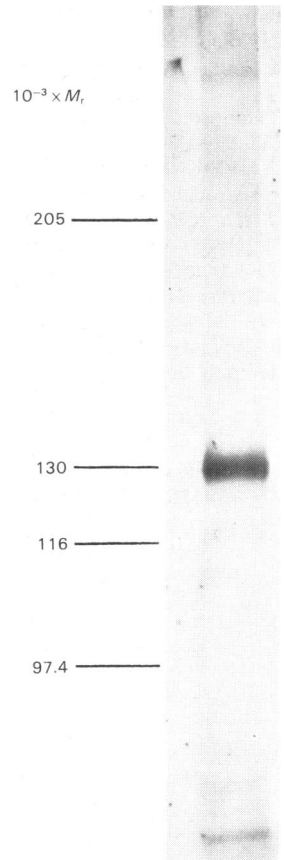

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of purified LDL-receptor protein*

Proteins were separated on a SDS/6%-polyacrylamide gel and silver-stained by the method of Merril *et al.* (1980).

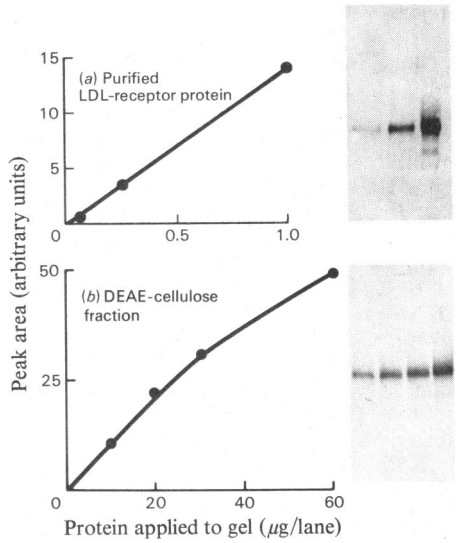

Fig. 5. *Quantification of LDL-receptor binding activity by ligand blotting with biotin–LDL*

Different amounts of (*a*) highly purified and (*b*) partially purified (DEAE-cellulose fraction) LDL-receptor protein were applied to separate SDS/polyacrylamide gels and blotted with biotin–LDL as described in the Materials and methods section. Black-and-white photographs of the gels were scanned with a densitometer, which computed the peak area (in arbitrary units). (*a*) Lanes 1–3, biotin–LDL ligand blots of purified LDL-receptor protein: 1, 0.06 μg of protein; 2, 0.25 μg of protein; 3, 1.0 μg of protein. (*b*) Lanes 1–4, biotin–LDL ligand blots of partially purified LDL-receptor protein (DEAE-cellulose fraction): 1, 10 μg of protein; 2, 20 μg of protein; 3, 30 μg of protein; 4, 60 μg of protein.

protein after SDS/polyacrylamide-gel electrophoresis under non-reducing conditions (Fig. 4), the density of the bands obtained by ligand blotting with biotin–LDL as determined by densitometric scanning was directly proportional to the amount of protein applied to the gel over a 20-fold range (Fig. 5*a*). Under these conditions a band was visible when only 60 ng of receptor protein was applied to the gel. Thus this method is at least as sensitive as the radioautographic method of Daniel *et al.* (1983).

When the partially purified DEAE-cellulose fraction of adrenal glands was applied to the gel, the band density (Fig. 5*b*) was also proportional to the amount of total protein applied to the gel over at least part of the range, suggesting that the method could be used to quantify LDL-receptor protein in cell extracts.

This new method for the detection of LDL receptors after electrophoresis and blotting has several advantages over previously published methods. It eliminates the need for specific antibodies against the ligand and for labelling of ligands and second antibodies with ^{125}I. Protein-bound ligand may be detected rapidly, since a single 30 min incubation with the streptavidin-detection reagent is required. Furthermore, since most of the plasma lipoproteins contain glycopeptides, biotin may be conjugated in this way to a variety of potential ligands for which specific antibodies are not available. The system could therefore be applied to the study of other lipoprotein receptors. Biotin-modified lipoproteins in combination with streptavidin-linked enzymes, fluorescent dyes or electron-dense particles may have useful applications in histochemistry or electron microscopy.

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