Immunoassay of bovine and human low-density-lipoprotein receptors using monoclonal antibodies

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Two methods are described for the assay of low-density-lipoprotein (LDL) receptor protein based on the binding of receptor to microtitre plate wells coated with a specific monoclonal antibody or with LDL, followed by detection with radioactive antibody that recognizes a different part of the molecule. The two-antibody procedure detected approx. 2 ng of pure bovine receptor at twice background, and there was a linear relationship on a double-logarithm plot between radioactive antibody bound and the amount of receptor added, up to at least 500 ng of receptor protein per well. The procedure using immobilized LDL was less sensitive and the binding of receptor was inhibited by low concentrations of NaCl, which restricted its usefulness for routine assay of tissue extracts. LDL receptor protein could be readily assayed using the two-antibody procedure in normal human skin fibroblast extracts prepared by bulk-elution from small columns of DEAE-cellulose followed by rapid desalting. No radioactive antibody bound with extracts of cells from a receptor-negative familial hypercholesterolaemic subject. The LDL receptor content of normal fibroblasts preincubated with lipoprotein-deficient serum was estimated, using bovine receptor as standard, to be approx. 60 ng of receptor protein/mg of cell protein.

INTRODUCTION

The LDL receptor is a glycoprotein expressed on the surface of cells which mediates the uptake and degradation of LDL, and is responsible for a significant proportion of LDL clearance from the circulation (Brown *et al.*, 1981). Although it has been suggested that LDL receptor activity, particularly in the liver, may be an important factor in determining the LDL concentration in plasma (Goldstein & Brown, 1984), the processes that regulate its expression are not fully understood. Methods for measuring LDL receptors in cells have depended on determining the amount of LDL bound to the surface of cells in culture (Goldstein *et al.*, 1976) or to isolated membranes prepared from whole tissue (Basu *et al.*, 1978). These measurements, especially with membranes, are often complicated by high blanks caused by non-specific binding (Harders-Spengel *et al.*, 1982). A direct assay of LDL receptor protein would provide a potentially useful complement to the functional assays, and, with the availability of monoclonal antibodies to the receptor (Beisiegel *et al.*, 1981; Soutar & Knight, 1986), has now become a possibility.

Immunoassays depending on competition for antibody require pure antigen for labelling and can be subject to interference when used with cell extracts. Although the LDL receptor can be purified (Schneider *et al.*, 1982), it is not easy to obtain in large quantities. 'Sandwich' assays, involving specific binding of antigen followed by detection with an antibody that recognizes a different part of the molecule, should be less susceptible to interference and only require pure antigen as a standard. In this paper we describe and evaluate two such assays for the LDL receptor, one employing LDL and an

antibody and the other employing two different antibodies.

METHODS

LDL (ρ 1.019–1.063 g/ml) and lipoprotein-deficient serum were prepared from fresh normal plasma as described previously (Wade *et al.*, 1985). LDL receptor protein was purified from bovine adrenal cortex by DEAE-cellulose chromatography (DEAE fraction) and affinity chromatography as described by Schneider *et al.* (1982). The protein concentration of the DEAE fraction was 760 μ g/ml and of the pure receptor preparation was 73 μ g/ml.

Monoclonal antibodies to the LDL receptor were isolated, purified and labelled with Na¹²⁵I as described by Soutar & Knight (1986). Three different antibodies were used in these studies. Antibody 9A3 recognized the bovine receptor, but not the human receptor. Antibodies 4B3 and 10A2 recognized both receptors and could be used to immunoprecipitate the human receptor protein from cell extracts, but, whereas antibody 10A2 bound to receptors expressed on the surface of cultured fibroblasts, antibody 4B3 did not (Soutar & Knight, 1986). Methods for immunoblotting and ligand blotting have been described previously (Soutar & Knight, 1986; Wade *et al.*, 1985).

Standard assays were developed which gave optimal conditions for the detection of LDL receptors. For the LDL-antibody procedure, wells of microtitre plates (flexible vinyl, flat-bottomed, from Dynatech Laboratories, Billingshurst, Sussex, U.K.) were first coated with

Abbreviations used: LDL, low-density lipoprotein; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate.

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LDL by incubation for 1 h at 37 °C with 100 μ l of a solution of LDL (500 μ g of protein/ml) in PBS (pH 7.4; Dulbecco & Vogt, 1954) containing 0.01% NaN_3 (PBS/azide). For the double-antibody assay, wells were coated by incubation for 1 h at 37 °C with 100 μ l of a solution of purified antibody (200 μ g/ml) in PBS/azide. The coated wells were washed for 3 $\times$ 3 min with PBS/azide containing 0.05% (v/v) Tween 20 (Sigma Chemical Co., Poole, Dorset, U.K.), incubated for 1 h at 37 °C with 200 μ l of 1% (w/v) bovine serum albumin in PBS/azide, and washed again as before. The wells were then incubated for 16 h at room temperature with 100 μ l of the sample or standard of LDL receptor protein in 50 mM-Tris/maleate buffer (pH 6.0) containing 2 mM- CaCl_2 and 30 mM-CHAPS (Sigma Chemical Co.). After washing as described above, wells were incubated for 1 h at 37 °C with ^{125}I -labelled antibody in PBS/azide/Tween (300 000 c.p.m./well; sp. radioactivity approx. 3000 c.p.m./ng). The plates were washed for 4 $\times$ 3 min each with PBS/azide/Tween, air-dried and cut up, and the individual wells were assayed for radioactivity.

In the initial experiments with bovine receptors, 9A3 was used as the immobilized antibody and 4B3 as the radioactive antibody. Later, to assay human receptors, antibody 10A2 was used instead of 9A3. Since antibody 4B3 could be purified in greater quantities, it was then used as the immobilized antibody with 10A2 as the labelled antibody.

Human skin fibroblasts were maintained in culture, seeded into 14 cm-diameter Petri dishes (2.5×10^5 cells/dish) and preincubated in medium containing lipoprotein-deficient serum (2.5 mg of protein/ml) as described previously (Knight & Soutar, 1982). Cells were washed twice with ice-cold PBS, scraped from the dish with a rubber blade and centrifuged at 100 g for 5 min at 4 °C. Cells from 15 dishes were pooled and washed once in PBS. Subsequent extraction and chromatography were performed at 4 °C. The cell pellet was partially solubilized in 4 ml of 50 mM-Tris/maleate buffer, pH 6.0, containing 2 mM- CaCl_2 , 50 mM- NaCl , 1% Triton X-100, 1 mM-phenylmethanesulphonyl fluoride and 0.1 mM-leupeptin, by ten strokes of a loose-fitting Teflon pestle in a small glass homogenizer tube. The extract was centrifuged at 100 000 g for 1 h at 4 °C in a Beckman 50.3 rotor. The supernatant was diluted with 12 ml of 50 mM-Tris/maleate buffer, pH 6.0, containing 2 mM- CaCl_2 and 1% Triton X-100, and applied to a 1 ml column of DEAE-cellulose (DE52; Whatman, Maidstone, Kent, U.K.) equilibrated in the same buffer in a 2 ml plastic syringe. The column was washed with 12 ml of 50 mM-Tris/maleate buffer, pH 6.0, containing 2 mM- CaCl_2 and 30 mM-CHAPS (CHAPS buffer). The syringe was suspended in a plastic tube and centrifuged for 5 min at 800 g . The cellulose was resuspended in 0.6 ml of the CHAPS buffer containing 500 mM- NaCl , the ends of the syringe were blocked and the syringe was placed on a rotary mixer for 15 min. The syringe was unblocked, suspended in a fresh plastic tube and re-centrifuged for 5 min at 800 g . The buffer containing the proteins eluted by the NaCl collected in the plastic tube. The eluate was rapidly desalted on a 5 ml column of Sephadex G-25 (Pharmacia Biotechnology, Uppsala, Sweden) swollen and equilibrated in CHAPS buffer. Samples were diluted, as required, in CHAPS buffer before being assayed for LDL receptor protein. The recovery of bovine receptors after ion-exchange chroma-

tography and desalting, as described, was $58 \pm 2\%$ (mean $\pm$ S.E.M. for five determinations).

RESULTS

Samples of partially purified LDL receptor from bovine adrenal cortex (DEAE fraction) were diluted in PBS and incubated in microtitre wells that had been coated with LDL. The wells were then incubated with ^{125}I -labelled monoclonal antibody that recognized a region of the LDL receptor distinct from the LDL-binding site. After exhaustive washing, radioactive antibody remained in the wells in amounts that were related to the concentration of DEAE fraction present during the initial incubation (Fig. 1). Wells incubated with samples of the same DEAE fraction that had been depleted of LDL receptors by passing through an affinity column of LDL-Sepharose retained much less radioactive antibody (Fig. 1).

These initial experiments indicated that solubilized LDL receptors could be bound by immobilized LDL in sufficient quantities to be detected by the subsequent addition of a specific antibody. When each was expressed as a logarithm, there was a linear relationship between the amount of radioactive antibody bound and the amount of DEAE fraction that had been added to the well. The slope of this line was greater when the incubation buffer was changed to Tris/maleate and included a detergent (Fig. 2a). Triton X-100, which had been used to solubilize the adrenal membranes, produced only a slight improvement. Octyl glucoside at the concentration present in the undiluted DEAE fraction was more effective, but the steepest slope was obtained with the zwitterionic detergent CHAPS. In contrast, the presence of NaCl , which is often used as an aid to

Fig. 1. Detection of LDL receptor protein in an extract of bovine adrenal cortex

Samples of bovine DEAE fraction, before (●) and after (○) passage through a column of LDL-Sepharose, were diluted to 100 μ l with PBS and incubated in wells coated with LDL. Bound LDL receptor protein was detected with radioactive antibody 9A3.

Fig. 2. Effect of detergents on the binding of LDL receptor protein to immobilized LDL (a) and immobilized anti-receptor antibody (b)

Bovine DEAE fraction was diluted with PBS (●) or 50 mM-Tris/maleate, pH 6.0, containing 2 mM- CaCl_2 with 30 mM-CHAPS (▲), 30 mM-CHAPS plus 0.5 M-NaCl (Δ), 40 mM-n-octyl glucoside (■), 1% Triton X-100 (▼) or 1% Triton X-100 plus 0.5 M-NaCl (○). Samples (100 μ l) of various dilutions were incubated in wells coated with LDL (a) or antibody 9A3 (b), and bound receptor was detected with radioactive antibody 4B3.

solubilization, decreased the amount of receptor bound. Studies with different concentrations of NaCl showed that 50% inhibition of LDL receptor binding to immobilized LDL in buffer containing 50 mM-Tris/maleate and 30 mM-CHAPS occurred with approx. 50 mM-NaCl. Binding was also inhibited by EDTA and suramin (50% inhibition with 1.5 mM and 0.3 mM respectively), both of which are known to prevent the binding of LDL to LDL receptors in membranes (Schneider *et al.*, 1982).

Micro-titre wells coated with monoclonal antibody to the LDL receptor, rather than with LDL, also bound protein present in the DEAE fraction that could be detected by using a second, radioactive, antibody which recognized a different part of the receptor molecule. Radioactivity bound was again related to the concentration of DEAE fraction added, and gave a straight line on a double-logarithm plot with a steeper slope than the assay with immobilized LDL (Fig. 2). Protein binding in this double-antibody assay was also affected by detergents (Fig. 2b). CHAPS and octyl glucoside were, as before, more effective than Triton X-100, but only altered the amount of protein bound and not the slope of the line. Unlike binding to LDL, binding to immobilized antibody was not particularly sensitive to salt. At all concentrations of receptor protein, binding in the presence of 30 mM-CHAPS was only decreased by approx. 35% on addition of 500 mM-NaCl (Fig. 2b).

Studies were performed with different concentrations of LDL and antibodies to determine the optimal conditions for the binding of receptor and radioactive antibody, which are given in the description of the standard assays in the Methods section. The best binding of receptor was obtained after an overnight incubation at room temperature. Shorter periods at 37 °C were less effective, and very little receptor was bound if the incubations were performed at 4 °C. Binding of receptors

to immobilized antibody gradually decreased if the pH of the incubation buffer was lowered below pH 6 (Fig. 3). In contrast, binding to immobilized LDL was much greater at pH 5 than at pH 6 (Fig. 3). However, it was not practical to use the lower pH, since cell extracts less pure than the bovine DEAE fraction precipitated during overnight incubations at pH 5 and gave unacceptably high blank values. Buffer of pH 6 was used for receptor binding in both assays, as this was the pH employed during DEAE-cellulose chromatography of cell extracts.

To confirm that the protein in the DEAE fraction

Fig. 3. Effect of pH on the binding of LDL receptor protein

Bovine DEAE fraction was diluted in 50 mM-Tris/maleate of the required pH containing 2 mM- CaCl_2 and 30 mM-CHAPS. Samples (100 μ l, containing approx. 1.2 μ l of DEAE fraction) were added to wells coated with LDL (■) or antibody 9A3 (●), and bound receptor was detected with radioactive antibody 4B3.

Fig. 4. Assay of bovine DEAE fraction against purified LDL receptor protein

DEAE fraction (▲) and a preparation of pure LDL receptor protein of known concentration (●) were suitably diluted in 50 mM-Tris/maleate, pH 6.0, containing 2 mM- CaCl_2 and 30 mM-CHAPS. Samples (100 μl) were incubated in wells coated with LDL (a) or antibody 9A3 (b), and bound receptor was detected with radioactive antibody 4B3. Results were corrected for background radioactivity observed in wells incubated with diluting buffer alone (1260 c.p.m. with immobilized LDL and 330 c.p.m. with immobilized antibody 9A3).

detected by the double-antibody assay was the LDL receptor, and to estimate its concentration, a highly purified preparation of receptors was obtained by affinity chromatography on a column of LDL coupled to Sepharose. Over 95% of the protein in this preparation, as judged from a silver stain after SDS/polyacrylamide-gel electrophoresis under non-reducing conditions, was present in a single band with an apparent M_r of 130000. This was the only protein detected by immunoblotting with the antibodies used in this study or by ligand blotting with biotin-labelled LDL (Wade *et al.*, 1985). Known amounts of pure LDL receptor were incubated

under standard assay conditions in wells coated with LDL or antibody 9A3, and bound receptor was detected with radioactive antibody 4B3. For each assay a double-logarithm plot of radioactivity bound against receptor protein added gave a straight line that was parallel with the line obtained with dilutions of the impure DEAE fraction (Fig. 4). At twice background, the double-antibody procedure detected approx. 2 ng of receptor protein, and the LDL-antibody procedure approx. 5 ng. By comparing radioactivity bound with that for the pure receptor, the amount of LDL receptor in the DEAE fraction could be estimated. The value given by the double-antibody assay was the same as that from the LDL-antibody assay. Results from six such determinations agreed well and gave a mean ($\pm$ S.E.M.) concentration of $27.4 \pm 0.8 \mu\text{g}$ of receptor protein/ml of DEAE fraction (range 24–30 $\mu\text{g}/\text{ml}$), or $3.59 \pm 0.11\%$ of the total protein.

To be of practical value, the assay should be sensitive and specific enough to detect receptors in a reasonably small sample of tissue with the minimum of purification. To explore this possibility, assays were performed with extracts from cultured human fibroblasts that had been preincubated with lipoprotein-deficient serum to induce the formation of receptors. Antibody 9A3 was found to be unable to recognize the human LDL receptor, and was replaced in these studies by antibody 10A2. Cell pellets were lysed with Triton X-100, and the LDL receptors were partially purified and concentrated by absorption and bulk elution from small columns of DEAE-cellulose. Different dilutions of the eluates were added to wells coated with antibody 4B3 and bound receptor was detected with radioactive antibody 10A2. When plotted as a logarithm against dilution, radioactivity bound by two normal cell extracts was on lines that were parallel with a standard curve prepared from the bovine DEAE fraction (Fig. 5). Wells incubated with extracts of fibroblasts from a receptor-negative homozygous familial-hypercholesterolaemic subject bound no more radioactivity than did blank wells incubated with buffer alone (Fig. 5). The addition of this extract to wells containing bovine receptor did not affect the amount of labelled antibody bound, suggesting that non-receptor proteins in the DEAE eluates did not influence the binding of the receptor to immobilized antibody.

Before the receptor content of human extracts could be estimated by comparison with a standard curve prepared with bovine receptor, it was necessary to check that the human receptor interacted in the same way as the bovine receptor with the two antibodies. Concentration curves showed that the apparent affinity of the human LDL receptor for radioactive antibody 10A2 was similar to that of the bovine receptor and that the concentration of antibody in the standard assay was sufficient to achieve maximum binding of both receptors. Furthermore, the concentration of antibody 4B3 used to coat the wells was high enough to give maximum binding of both receptors. However, it was still possible that the same amount of immobilized antibody could bind different amounts of human and bovine receptors. Since it was unlikely that immobilized LDL would show a similar difference, the same extract from normal cells was assayed by both the double-antibody procedure and the LDL-antibody procedure. The extract was prepared by lysis and DEAE-cellulose chromatography, as before, and was desalted on a column of Sephadex G-25. Radioactivity

Fig. 5. Detection of LDL receptor protein in extracts from cultured human fibroblasts preincubated with lipoprotein-deficient serum

Cells from two normal subjects (□, ■) and a receptor-negative familial-hypercholesterolaemic subject (▼) were solubilized and chromatographed on DEAE-cellulose as described in the Methods section. The eluate was diluted in eluting buffer (50 mM-Tris/maleate, pH 6.0, containing 2 mM- CaCl_2 , 30 mM-CHAPS and 500 mM-NaCl), and 100 μl samples were incubated in wells coated with antibody 4B3. Standards of bovine DEAE fraction of known LDL receptor protein content (●) were diluted in the same eluting buffer. Background radioactivity in wells incubated with eluting buffer alone was 650 c.p.m./well.

bound was corrected for background binding by using values obtained with the same samples in the presence of 5 mM-suramin for the LDL-antibody assay and buffer alone for the double-antibody assay. Little radioactivity was bound if antibody 9A3 was used as the immobilized antibody with the human receptor, suggesting that non-specific binding in the double-antibody assay was negligible. The amount of receptor protein in the extract was determined by comparison with a standard curve prepared from a known amount of bovine DEAE fraction that had been subjected to the same ion-exchange and gel-filtration procedures. Estimates of receptor protein by the double-antibody assay were the same as those from the LDL-antibody assay and were directly related to the concentration of extract added (Fig. 6).

The receptor concentration of extracts of fibroblasts from three different normal subjects was assayed by the double-antibody procedure. Each was corrected for losses during ion-exchange chromatography and gel-filtration, by using the recovery of a known amount of bovine DEAE fraction run at the same time (59%, 51% and 56% respectively for the results given below), and was expressed as a function of the protein present in the total cell lysate. The values obtained for the LDL receptor content of fibroblasts that had been preincubated with lipoprotein-deficient serum were 62, 43 and 69 ng of LDL receptor protein/mg of cell protein.

Fig. 6. Comparison of assays of human LDL receptor by the LDL-antibody procedure and the double-antibody procedure

An extract from cultured normal human fibroblasts preincubated with lipoprotein-deficient serum was prepared by ion-exchange chromatography and gel filtration as described in the Methods section. It was diluted in 50 mM-Tris/maleate buffer, pH 6.0, containing 2 mM- CaCl_2 and 30 mM-CHAPS, and samples (100 μl) were incubated in wells coated with LDL (●), antibody 4B3 (■) or antibody 9A3 (▲). LDL receptor protein was detected with radioactive antibody 10A2. Radioactivity bound was corrected for background binding observed in the presence of 5 mM-suramin for the LDL-antibody assay and with diluting buffer alone for the double-antibody assays. Values of receptor protein were obtained by comparison with a standard curve prepared under the same conditions from a sample of bovine DEAE fraction (containing 820 ng of receptor protein) that had been subjected to ion-exchange chromatography and gel filtration at the same time as the fibroblast extract.

DISCUSSION

The protein in impure extracts that bound to immobilized LDL and was detected with an antibody to the LDL receptor behaved in all aspects like the authentic receptor. Binding was inhibited by EDTA and suramin and the protein was removed by an affinity column that bound the receptor. Furthermore, binding was greater in the presence of detergents and at low pH, which would be expected for an acidic protein that normally functions in cell membranes. The inhibition of the binding of solubilized receptors to immobilized LDL by low concentrations of NaCl was unexpected. This, together with the relatively low sensitivity of the method, rendered the LDL-antibody procedure unsuitable for the routine measurement of receptor protein in extracts from

small amounts of tissue prepared by using a rapid, but not completely efficient, desalting column. However, the procedure could be employed under carefully controlled conditions with appropriate blanks and standards to discover whether the receptors in a particular extract had the ability to bind LDL.

A variety of evidence indicated that the protein detected by the double-antibody procedure was the LDL receptor. First, the antibodies were raised against an almost pure preparation of LDL receptors and, when used to immunoblot impure DEAE fractions, detected only a single protein with the M_r expected for the LDL receptor (Daniel *et al.*, 1983). Secondly, the receptor content of the DEAE fraction estimated from standards of the pure receptor was the same as that obtained with the LDL-antibody assay. Finally, no protein was detected in extracts of fibroblasts from a receptor-negative homozygous familial-hypercholesterolaemic subject. Assays with direct extracts of cells or membranes were subjected to interference by components that were removed by ion-exchange chromatography. Although receptor protein could be detected in the eluates from DEAE-cellulose, the presence of salt slightly but significantly inhibited binding, and samples were normally desalted on small columns of Sephadex G-25 before assay. Non-receptor proteins in those extracts did not apparently influence the values obtained. Thus reliable assays could be performed on cell extracts after a simple bulk-elution and desalting procedure, which gave over 50% recovery and a beneficial concentration of receptor protein.

The LDL-receptor content of human fibroblasts preincubated in lipoprotein-deficient serum was estimated by the double-antibody assay to be approx. 60 ng of protein/mg of cell protein. If one receptor bound one particle of LDL, this amount of receptor would bind approx. 300 ng of LDL protein. Monolayers of fibroblasts under the same conditions bound about 100 ng of LDL protein/mg of cell protein. Thus, since not all of the receptors are expected to be on the cell surface at any one time (Wileman *et al.*, 1985), the assay of receptor content agreed reasonably well with that predicted from direct binding experiments, and suggests that the seven repeated sequences observed in the probable binding region of the receptor (Sudhof *et al.*, 1985) do not each bind one particle of LDL. The available evidence indicates that human and bovine receptors behaved similarly in the assays. Conditions were optimal for each and the two assays gave the same results. However, until a sample of pure human receptor of known protein concentration is available, a slight doubt must still remain about the absolute values for human receptors obtained by using a standard from bovine adrenals.

The double-antibody procedure could reliably assay

the concentration of LDL receptors in extracts from two 14 cm-diameter dishes of fibroblasts and is sensitive enough to allow controlled experiments on the synthesis and breakdown of receptor protein in cultured cells. Since both antibodies also recognize the precursor form of the receptor (Soutar & Knight, 1986), the assay could also be used to study the regulation of precursor production in cells from those homozygous familial-hypercholesterolaemic subjects who are unable to process the protein further (Goldstein & Brown, 1984). Estimates of relative receptor content obtained from ligand-blotting experiments (A. K. Soutar, D. P. Wade, K. Harders-Spengel & B. L. Knight, unpublished work) indicate that LDL receptor protein could be assayed in samples of human liver weighing about 1 g. Thus, by using the appropriate antibodies for the species studied, the methods described in this paper should be able to provide the basis for assays with human and animal tissues that will provide further insight into the mechanisms that control the expression of LDL receptors *in vivo*.

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