Volume 230, number 1,2, 176–180 FEB 05713 March 1988

Demonstration of a high density lipoprotein (HDL)-binding protein in Hep G2 cells using colloidal gold-HDL conjugates

Ambrosios M. Kambouris, Paul D. Roach and Paul J. Nestel

CSIRO, Division of Human Nutrition, Kintore Avenue, Adelaide, SA 5000, Australia

Received 25 January 1988

Solubilized membrane proteins of Hep G2 cells were electrophoretically separated on polyacrylamide gels and electrotransferred onto nitrocellulose paper. Overlaying the nitrocellulose with human high density lipoproteins conjugated to colloidal gold revealed the presence of a single protein band with an apparent molecular mass of 80 kDa. Binding of the conjugates to this protein was specific for high density lipoproteins in as much as it was effectively displaced by an excess of unlabelled high density lipoproteins but not by a similar excess of unlabelled low density lipoproteins. Binding was not dependent on Ca^{2+} as 10 mM EDTA had no effect. The binding activity of the solubilized membranes was increased by incubating the cells with non-lipoprotein cholesterol. This was detected on electroblots and quantified with a new dot blot assay using the colloidal gold-high density lipoprotein conjugates.

HDL receptor; Colloidal gold; Electroblotting; Dot blotting; Ligand blotting; (Hep G2 cell)

1. INTRODUCTION

High density lipoproteins are crucial to the orderly metabolism of cellular cholesterol. This involves transport of cholesterol between organs which in turn requires regulated interaction between cells and HDL. The demonstration of high-affinity binding sites for HDL apoproteins A-I, A-II and A-IV [1–3] which appear to mediate either the delivery of cholesterol to cells [4], or the transfer of cholesterol out of cells [5,6], favours the presence of specific receptors. This has been strengthened by the demonstration of proteins from membranes of several organs that bind specifically either to HDL_3 (which contains no apoprotein E), or to the HDL apoproteins. However, several apparently distinct proteins have been reported. The first, from sheep adrenal, rat liver and rat kidney is an 80 kDa protein [7,8] whereas that from human placenta is larger (120 kDa) [9]. Graham and Oram [10] have identified a 110 kDa protein in membranes of several species of cells which binds HDL and is regulated by intracellular cholesterol levels.

We describe in this report a new technique for detecting and quantifying the activity of the HDL_3 binding protein. It is based on the recently published and highly sensitive method of Roach et al. [11] for the detection of the LDL receptor using colloidal gold-complexed lipoproteins and the amplification of the colour by silver staining. Adapting this technique for HDL, we have demonstrated a HDL-binding protein from the human hepatoma cell line, Hep G2, using colloidal gold-HDL_3 conjugates. Furthermore, we have shown by electroblotting and quantitative dot blotting that this protein is up-regulated by incubating the cells in the presence of cholesterol.

Correspondence address: A.M. Kambouris, CSIRO, Division of Human Nutrition, Kintore Ave., Adelaide, SA 5000, Australia

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; MEM, minimal essential medium; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin

Published by Elsevier Science Publishers B.V. (Biomedical Division)

2. MATERIALS AND METHODS

Lipoproteins (HDL_3, d = 1.125–1.21 g/ml and LDL, d = 1.025–1.05 g/ml) were obtained from normolipemic human plasma by preparative ultracentrifugation [12] (including a wash to remove albumin), dialyzed against 0.15 M NaCl, pH 7.4, sterilized using a 0.45 μm Millipore filter and used within 2 weeks of preparation.

Colloidal gold was prepared by the method of Frens [13]: 2.8 ml of 1% (w/v) trisodium citrate were added to 100 ml of a 0.01% (w/v) $HAuCl_4$ boiling solution and the boiling was continued under reflux until the colour became red and did not change.

HDL_3 was conjugated to colloidal gold essentially as described for LDL [14]; 5 ml of colloidal gold was jetted into a tube containing 80 μg of HDL_3 protein diluted to a volume of 0.5 ml with deionized water. Conjugates were pelleted by centrifugation at room temperature at 10000 × g for 20 min in Bunzel polypropylene centrifuge tubes and washed once in deionized water under the same conditions. Conjugates were prepared fresh when required.

The human hepatoma cell line, Hep G2, was grown in minimal essential medium (MEM) supplemented with 10 ml/l of 100 × MEM vitamins, 0.11 g/l of sodium pyruvate, 1.17 g/l of L-glutamine and 10% (v/v) fetal calf serum. To increase the cellular concentration of free cholesterol, confluent monolayers were washed with PBS containing 1 mg/ml fatty acid free albumin and further incubated with culture medium containing non-lipoprotein cholesterol (5–50 μg/ml) and progesterone (20 μg/ml) as described by Oram [15].

Preparation and solubilization of cellular membranes was essentially as described elsewhere for rat livers [16]. The Hep G2 cells were scraped from the culture flasks, supplemented in 20 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM PMSF and homogenized for 2 periods of 15 s with a Janke and Kinkel Ultra Tarrax (Staufen, FRG) set at 10. The 3000–100000 × g fraction was prepared by ultracentrifugation and the pellet was suspended in 0.25 mM Tris-maleate, pH 6.5, containing 1 mM PMSF and sonicated for 2 periods of 20 s using a Branson sonifier (Danbus, USA) set at 4. Triton X-100 was then added to a final concentration of 1% (w/v). Insoluble material was removed by centrifugation at 100000 × g for 1 h at 4°C and the detergent was removed using Amberlite XAD-2 [16]. Finally, the insoluble debris was removed by centrifugation at 10000 × g for 10 min and the solution kept at 4°C for up to 2 weeks or frozen at −80°C.

Unreduced, unheated solubilized membrane proteins (100 μg/well) were separated by electrophoresis on polyacrylamide gels containing SDS [17] and electrotransferred onto nitrocellulose paper according to Burnette [18] using a water cooled Bio-Rad apparatus for 18 h at 180 mA. Gels were calibrated with either Bio-Rad or Pharmacia low molecular mass protein standards.

For dot blot assays, the solubilized Hep G2 cell membrane proteins were spotted onto nitrocellulose paper using a Bio-Rad dot blot apparatus. Samples (2 μg) were applied in 20 μl of 125 mM Tris-maleate, pH 6.5, containing 1 mM PMSF and allowed to filter through the nitrocellulose paper by gravity at room temperature.

Detection of HDL_3 binding activity on electroblots and dot blots was done essentially as described for LDL [11]. The nitrocellulose strips were first incubated in quenching buffer (60 mM Tris-HCl, pH 8.0, containing 25 mM NaCl and 40 mg/ml BSA) for 1 h at room temperature and subsequently for 1 h in incubation buffer (60 mM Tris-HCl, pH 8.0, containing 25 mM NaCl, 10 mM EDTA and 20 mg/ml BSA) containing 20 μg/ml colloidal gold-HDL_3 conjugates. For competition studies, a 25-fold excess of unlabelled HDL_3 or LDL was included in the incubation buffer along with the conjugated HDL_3. After the gold-ligand overlay, the nitrocellulose strips

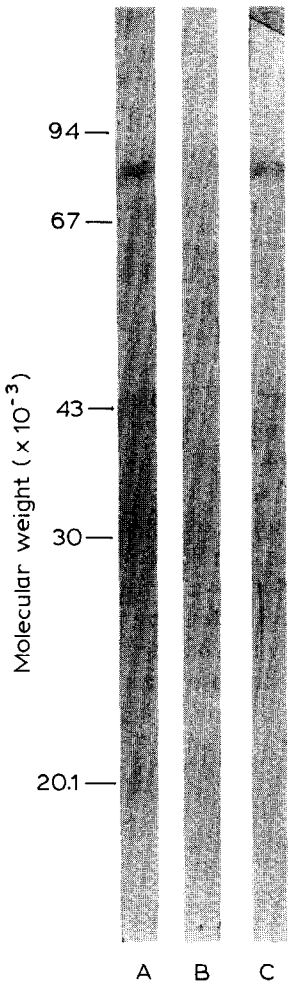

Fig.1. Competitive displacement of colloidal gold-HDL_3 conjugate binding to a protein band on nitrocellulose. Solubilized membrane proteins from Hep G2 cells, 100 μg, were subjected to electrophoresis and electroblotted onto nitrocellulose paper. The electroblots were incubated with 20 μg/ml of colloidal gold-HDL_3 conjugates only (A), in the presence of a 25-fold excess of unlabelled HDL_3 (B) or a similar excess of unlabelled LDL (C). The nitrocellulose strips were subsequently incubated in silver stain.

were rinsed extensively with deionized water then with 20 mM citrate buffer, pH 3.85, before being silver stained [19] as described [11]. For quantitation, the dot blots were soaked in toluene to render them transparent and scanned using an LKB 2202 ultrascan laser densitometer interfaced to a Hewlett Packard computing integrator.

3. RESULTS

Among the solubilized membrane proteins of Hep G2 cells, only one protein interacted with the gold-HDL_3 after electrophoresis through a polyacrylamide gel and electrotransfer onto nitrocellulose. Lane A of fig.1 shows the single band which was visualized after overlaying with the HDL_3 conjugates. This band corresponded to a protein with an apparent molecular mass of about 80 kDa. The interaction of the conjugates with this protein is specific for HDL_3 in that the gold-HDL_3 was displaced by a 25-fold excess of unlabelled HDL_3 (fig.1, lane B) but not by a similar excess of unlabelled LDL (fig.1, lane C).

The regulation of the expression of this protein in response to the cholesterol content of the medium is shown in fig.2. The binding of gold-HDL_3 to the 80 kDa membrane protein increased when Hep G2 cells were incubated for 24 h in medium containing between 5 and 50 μg/ml of non-lipoprotein cholesterol. The increase in cellular cholesterol was entirely in the unesterified fraction. The HDL binding protein appeared to be up-regulated by incubating the cells in the presence of cholesterol because the coloration of the band

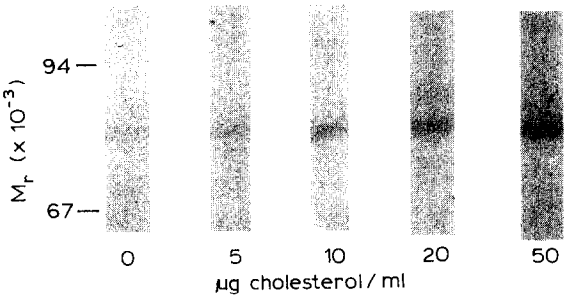

Fig.2. The effect of cholesterol on the HDL binding protein. Solubilized membrane proteins, 100 μg, from Hep G2 cells incubated in the presence of the indicated amount of cholesterol were subjected to electrophoresis and electroblotted onto nitrocellulose paper. The electroblots were incubated with 20 μg/ml of colloidal gold-HDL_3 conjugates and subsequently incubated in silver stain.

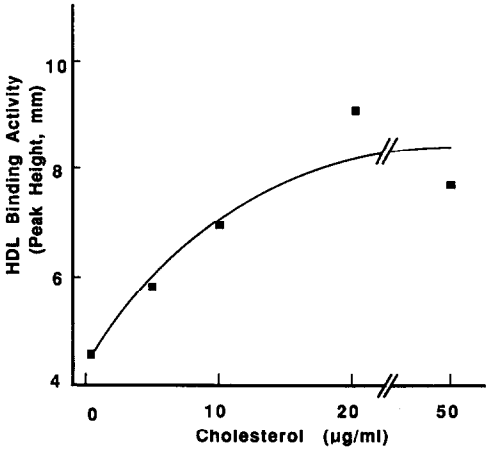

Fig.3. The effect of cholesterol on the specific HDL binding activity of Hep G2 cells measured by the dot blot assay. Solubilized membrane proteins (2 μg) from cells incubated in the presence of the indicated amount of cholesterol were dot blotted onto nitrocellulose and incubated with 20 μg/ml of colloidal gold-HDL_3 conjugates in the absence (total binding) and presence (nonspecific binding) of 1 mg/ml unlabelled HDL_3. The nitrocellulose was then silver stained and scanned with a laser densitometer interfaced to a computing integrator. The specific binding was obtained by subtracting the nonspecific binding from the total binding and expressed as integrator peak height in mm.

was more intense for cells incubated in the cholesterol-containing medium than for cells incubated in the absence of cholesterol (fig.2). This finding was confirmed with the dot blot assay. The HDL_3 binding activity increased in a saturable manner with the amount of cholesterol in the medium (fig.3). Up to a two-fold increase in specific HDL_3 binding was observed.

4. DISCUSSION

This study demonstrates that the protein from Hep G2 cells which binds HDL_3 specifically is sensitively regulated by cellular free cholesterol. The binding of the gold-labelled HDL_3 was effectively displaced by excess unlabelled HDL_3 but not by unlabelled LDL and the protein differed from the LDL receptor in having a low molecular mass: 80 kDa vs 130 kDa for the LDL receptor under similar electrophoretic conditions [20]. Also unlike the binding to the LDL receptor, the binding of HDL_3 to this protein is independent of Ca^{2+}; the

coloration was observed in the presence of 10 mM EDTA.

Hep G2 cells have been shown by Dashti et al. [21] to bind and degrade human ^{125}I-HDL. They however found the interaction to be rather non-specific in that human unlabelled VLDL and LDL were as effective in displacing ^{125}I-HDL as unlabelled HDL. In contrast, Hoeg et al. [22] found that radioiodinated apo E-free HDL interacted in a specific manner with Hep G2 cells as well as with membranes from human liver biopsies; unlabelled LDL was not able to displace the binding of ^{125}I-HDL.

The molecular masses of the cellular proteins that bind HDL have been reported to vary between 80 and 120 kDa and in this study appeared to be approx. 80 kDa. It remains to be seen whether there are indeed multiple distinct proteins that specifically bind HDL or whether the smaller proteins represent biologically active degradation fragments.

The findings with the cholesterol-incubated Hep G2 cells are a direct demonstration of the physiological role of the putative HDL receptor and the sensitivity of our technique to detect a difference in its expression. The increase in the 80 kDa protein is entirely consistent with the greater binding of ^{125}I-HDL$_3$ to these cells observed by Hoeg et al. [22] following incubation with cholesterol. Similar results have also been reported for the binding of ^{125}I-HDL to cholesterol loaded fibroblasts and arterial smooth muscle cells [23]. Clearly, the expression of the HDL binding protein is stimulated in cells when there is a need to transfer excess cholesterol out of the cell. However, HDL binding activity also increases when rat adrenocortical cells are stimulated to raise corticosteroid production, a process requiring cholesterol [24].

The up-regulation of the putative HDL receptor is in contrast to the down-regulation of the LDL receptor others [25] and ourselves (not shown) have observed in Hep G2 cells incubated with cholesterol. These reciprocal changes may however be regulated via a common mechanism and the concentration of unesterified cholesterol is most likely to play a role. We have also recently observed a similar reciprocal relationship between the LDL and HDL receptor activities in the livers of rats fed fish oil [26]. The concentration of unesterified cholesterol in the livers of these rats may well rise because the production and secretion of VLDL [27] and therefore the secretion of cholesterol is severely inhibited by the dietary oils. The livers of the fish oil fed-rats, like the Hep G2 cells incubated with cholesterol, probably attempt to decrease the concentration of unesterified cholesterol by down-regulating their LDL receptors to reduce the uptake of cholesterol-rich LDL [28]. It is also tempting to suggest that up-regulating the putative HDL receptor may lead to an enhanced efflux of cholesterol from these livers and Hep G2 cells in keeping with the involvement of HDL in the efflux of cholesterol from peripheral cells in culture [5,6].

Acknowledgements: We would like to thank Alison Tuckfield and Kath Illes for preparation of the manuscript. P.D.R. is a post-doctoral fellow of the Canadian Heart Foundation. This work was supported by the Australian National Heart Foundation.

REFERENCES

[1] Fidge, N.H. and Nestel, P.J. (1985) J. Biol. Chem. 260, 3570–3575.
[2] Chacko, G.K. (1982) Biochim. Biophys. Acta 712, 129–141.
[3] Dvorin, E., Gorder, N.L., Benson, D.M. and Gotto, A.M. jr (1986) J. Biol. Chem. 261, 15714–15718.
[4] Glass, C., Pittman, R.C., Civen, M. and Steinberg, D. (1985) J. Biol. Chem. 260, 744–750.
[5] Ho, Y.K., Brown, M.S. and Goldstein, J.L. (1980) J. Lipid Res. 21, 391–398.
[6] Wu, J.D. and Bailey, J.M. (1980) Arch. Biochem. Biophys. 202, 467–473.
[7] Fidge, N., Kagami, A. and O'Connor, M. (1985) Biochem. Biophys. Res. Commun. 129, 759–765.
[8] Fidge, N.H. (1986) FEBS Lett. 199, 265–268.
[9] Keso, L., Lukka, M., Ehnholm, C., Baumann, M., Vihko, P. and Olkinvora, M. (1987) FEBS Lett. 215, 105–108.
[10] Graham, D.L. and Olam, J.F. (1987) J. Biol. Chem. 262, 7439–7442.
[11] Roach, P.D., Zollinger, M. and Noël, S.P. (1987) J. Lipid Res. 28, 1515–1521.
[12] Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345–1353.
[13] Frens, G. (1973) Nature Phys. Sci. 241, 20–22.
[14] Handley, D.A., Arbeeney, C.M., Witte, L.D. and Chien, S. (1981) Proc. Natl. Acad. Sci. USA 78, 368–371.
[15] Oram, J.F. (1986) Methods Enzymol. 129, 645–659.
[16] Roach, P.D. and Noël, S.P. (1985) J. Lipid Res. 26, 713–720.

[17] Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods Enzymol. 26, 3–27.
[18] Burnett, W.N. (1981) Anal. Biochem. 112, 195–203.
[19] Danscher, G. (1981) Histochem. 71, 81–88.
[20] Havinga, J.R., Lohse, P. and Beisiegel, U. (1987) FEBS Lett. 216, 275–280.
[21] Dashti, N., Wolfbauer, G. and Alaupovic, P. (1985) Biochim. Biophys. Acta 833, 100–110.
[22] Hoeg, J.M., Demosky, S.J., Edge, S.B., Gregg, R.E., Osborne, J.C. jr and Brewer, H.B. jr (1985) Arteriosclerosis 5, 228–237.
[23] Oram, J.F., Brinton, E.A. and Bierman, E.C. (1983) J. Clin. Invest. 72, 1611–1621.
[24] Fidge, N., Leonard-Kanevsky, M. and Nestel, P. (1984) Biochim. Biophys. Acta 793, 180–186.
[25] Leichtner, A.M., Krieger, M. and Schwarts, A.L. (1984) Hepatology 4, 1274–1278.
[26] Roach, P.D., Kambouris, A.M., Trimble, R.P., Topping, D.L. and Nestel, P.J. (1987) FEBS Lett. 222, 159–162.
[27] Wong, S.H., Nestel, P.J., Trimble, R.P., Storer, G.B., Illman, R.J. and Topping, D.L. (1984) Biochim. Biophys. Acta 792, 103–109.
[28] Goldstein, J.L. and Brown, M.S. (1977) Annu. Rev. Biochem. 46, 897–930.