**High Density Lipoprotein–Binding Proteins in Porcine Liver**

**Isolation and Histological Localization**

Rini P.G. de Crom, Rien van Haperen, Rob Willemsen, and Arthur W.M. van der Kamp

The antiatherogenic properties of high density lipoproteins (HDLs) are thought to reside in their involvement in the reverse cholesterol transport pathway. Specific HDL-binding proteins could play a key role in this process. Two HDL-binding proteins of approximately 90 and 180 kd were identified in porcine liver by ligand blotting and were purified to apparent homogeneity by a combination of protein extraction, DEAE-cellulose chromatography, Con A-Sepharose chromatography, and preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Binding of $^{125}$I-HDL by these proteins could be actively competed for by unlabeled HDL but not by low density lipoprotein. Polyclonal antisera have been raised against these two proteins. Each antiserum recognized only one of the HDL-binding proteins, indicating that they are not immunologically related. Moreover, striking differences in localization were observed in immunohistochemical studies. The 90-kd protein is located within the hepatocellular plates, while the 180-kd protein is present along the lining of the sinusoids. These results suggest functional differences between the two HDL-binding proteins described.

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**KEY WORDS** • high density lipoproteins • high density lipoprotein–binding proteins • porcine liver • immunohistochemical localization

High density lipoproteins (HDLs) are widely believed to play a protective role in the development of coronary heart disease (CHD). This hypothesis is based on epidemiological data, which show a strong inverse correlation between plasma HDL cholesterol level and the prevalence of CHD, and on intervention studies, which indicate that elevation of HDL cholesterol level is effective in the primary prevention of CHD. The antiatherogenic properties of HDL are probably based on its ability to promote efflux of cholesterol from peripheral cells and to deliver it to the liver for excretion, a concept termed reverse cholesterol transport.

The binding characteristics of HDL to cultured cells and to purified plasma membrane fractions strongly suggest the existence of a specific, high-affinity HDL receptor. Because such a receptor could have a key position in the process of reverse cholesterol transport, several groups have sought to identify HDL-binding proteins by using ligand blot studies. Their results differ in the molecular masses of HDL-binding proteins found, which may be attributable to species and/or tissue specificity.

It is conceivable that in the reverse cholesterol transport pathway, the tissues involved in cholesterol efflux (peripheral tissues) have a different HDL metabolism than the liver, which is involved in cholesterol clearance. A peripheral HDL-binding protein has been described and characterized by Oram and coworkers. We investigated HDL-binding proteins in the liver. In the present study, two HDL-binding proteins in porcine liver are described. Their histological localization is notably different, presumably reflecting their involvement in different steps in the reverse cholesterol transport pathway.

**Methods**

**Lipoproteins**

Low density lipoproteins (LDLs) ($d=1.019–1.063$ g/ml) and HDL ($d=1.063–1.21$ g/ml) were isolated from human plasma of healthy volunteers by sequential ultracentrifugation. The same procedure was followed for the isolation of porcine lipoproteins. HDL was subjected to heparin-Sepharose affinity chromatography to remove apolipoprotein B/E-containing particles. Lipoproteins were iodinated by the iodine monochloride method. Specific activities were about 300 cpm/ng protein. All studies were performed with human lipoproteins, unless indicated otherwise.

**Ligand Blot Assay**

Samples of protein fractions were dialyzed against 5 mM NH$_4$HCO$_3$ and 1 mM EDTA and were concentrated by lyophilization. Protein fractions were electrophoresed on 7% polyacrylamide slab gels in the presence of sodium dodecyl sulfate (SDS-PAGE) under nonreducing conditions and electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.; 0.45-$\mu$m). Nitrocellulose strips were incubated for 2 hours with blocking buffer (10 mM...
tris(hydroxymethyl)aminomethane [Tris] HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl₂, 1% [wt/vol] bovine serum albumin, 1% [wt/vol] low-fat milk powder [a gift from Nutricia B.V., Zoetermeer, The Netherlands] containing 50 μg/ml LDL, followed by a 2-hour incubation with blocking buffer containing 125 μg/ml LDL and 25 μg/ml HDL. Subsequently, strips were washed with blocking buffer followed by a short wash in 10 mM Tris HCl (pH 8.0), 150 mM NaCl, and 2 mM CaCl₂ (buffer A) and incubated for 10 minutes with buffer A containing 0.1% glutaraldehyde. HDL was visualized using rabbit anti-human HDL antiserum and peroxidase-conjugated, swine anti-rabbit immunoglobulins (Dako, Copenhagen, Denmark) as the secondary antibody. Alternatively, blots were incubated with blocking buffer as described, followed by an incubation with blocking buffer containing 10 μg/ml 125I-HDL. Blots were washed extensively in blocking buffer. After drying, protein bands were visualized by autoradiography.

Two-Dimensional Gel Electrophoresis

Two-dimensional PAGE was carried out according to the procedure of O'Farrell.²⁷

Solubilization of Liver Proteins

All operations were carried out at 0-4°C. Porcine livers obtained from the Department of Experimental Cardiology, Erasmus University Rotterdam, were collected on ice immediately after death of the animal, cut into small pieces, and stored at -70°C. For protein solubilization, 100 g liver tissue was minced using surgical blades and homogenized in 20 ml 10 mM Tris HCl (pH 8.0), 150 mM NaCl, 1 mM benzamidine, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (buffer B), using an Ultra-Turrax TP 18-10. Tissue debris was removed by centrifugation (10 minutes, 1,350g). The supernatant was centrifuged (60 minutes, 100,000g) and the pellet resuspended in 120 ml buffer B containing 1% (vol/vol) Triton X-100 by aspiration with a 22-gauge needle. The suspension was stirred for 10 minutes and slowly diluted 10 times in 10 mM Tris HCl (pH 8.0), 2 mM CaCl₂, and 1% (vol/vol) Triton X-100. After removal of particulate material by centrifugation (30 minutes, 20,000g), a clear supernatant was obtained.

DEAE-Cellulose Chromatography

The solubilized fraction was applied to a DEAE-cellulose (DE-52; Whatman, Clifton, N.J.) column (2.4×10 cm) at a flow rate of 30 ml/hr. The column was washed with 10 mM Tris HCl (pH 8.0), 2 mM CaCl₂, and 15 mM CHAPS (3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate) (buffer C). Elution was performed with buffer C containing increasing concentrations of NaCl.

Con A-Sepharose Chromatography

DEAE-cellulose fractions containing HDL-binding activity were pooled and dialyzed against 20 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7.2) containing 150 mM NaCl. To 400 μg of Con A-Sepharose eluate, various amounts of anti-HDL-binding protein antiserum were added as indicated in the legend to Figure 5 and incubated for 16 hours at 4°C. One-hundred fifty microliters of a 1:1 suspension of protein A-Sepharose (Pharmacia) was added, and the mixture was incubated for 2 hours at room temperature. After centrifugation (5 minutes, 400g), the supernatant was separated from the immunoprecipitate. Both were boiled in SDS-PAGE sample buffer and analyzed in the ligand blot assay.

Immunohistochemistry

Porcine liver was fixed and prepared for ultracytometry as described before.³⁰ Semithin cryosections (0.5-1.0 μm) were incubated with polyclonal antibodies against HDL-binding proteins. Antigen-antibody complexes were visualized with fluorescein isothiocyanate–conjugated goat

Preparative SDS-PAGE

Proteins eluted from Con A-Sepharose were subjected to SDS-PAGE. A small vertical strip of the gel was analyzed in the ligand blot assay. With this as a guide, HDL-binding proteins were cut out and extracted by electroelution. These proteins were subjected to another round of preparative SDS-PAGE until they appeared to be homogeneous on silver-stained gels. After transfer onto nitrocellulose membranes, a small horizontal strip containing the HDL-binding activity was cut out. After solubilizing the nitrocellulose with dimethyl sulfoxide, this fraction was used to immunize the rabbits.

Antisera

Antibodies to HDL or HDL-binding proteins were prepared by standard techniques. In brief, 50 μg protein was added to 1 ml phosphate-buffered saline and 1 ml incomplete Freund’s adjuvant. A rabbit (New Zealand White) was injected subcutaneously with this mixture. Two boosters with the same mixture were given 15 and 25 days after the first injection. Eight days later, the blood was collected and serum was obtained by centrifugation at 400g after clotting. All animals were cared for in compliance with the Guidelines of the Ethical Committee on Use of Laboratory Animals of the Erasmus University Rotterdam (1288G55) and with the European Committee Standards on the care and use of laboratory animals (Ministry of Welfare, Health and Cultural Affairs, The Netherlands).

Immunoblotting

Protein samples were subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. These were incubated with blocking buffer as described for the ligand blot assay procedure followed by a 2-hour incubation with blocking buffer containing 0.05% (vol/vol) Tween-20 and anti-HDL-binding protein antiserum (dilution, 1:1,000). Peroxidase-conjugated, swine anti-rabbit immunoglobulins (Dako) were used as the secondary antibody.

Immunoprecipitation

Con A-Sepharose eluate was dialyzed against 20 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7.2) containing 150 mM NaCl. To 400 μg of Con A-Sepharose eluate, various amounts of anti-HDL-binding protein antiserum were added as indicated in the legend to Figure 5 and incubated for 16 hours at 4°C. One-hundred fifty microliters of a 1:1 suspension of protein A-Sepharose (Pharmacia) was added, and the mixture was incubated for 2 hours at room temperature. After centrifugation (5 minutes, 400g), the supernatant was separated from the immunoprecipitate. Both were boiled in SDS-PAGE sample buffer and analyzed in the ligand blot assay.

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FIGURE 1. DEAE-cellulose chromatography. Proteins were solubilized from porcine liver and applied to DEAE-cellulose. Fractions were eluted with the following concentrations of NaCl: 50 mM (lanes 1 and 2); 75 mM (lanes 3 and 4); 100 mM (lanes 5 and 6); and 125 mM (lanes 7 and 8). Samples of these fractions were subjected to the high density lipoprotein (HDL) ligand blot assay. In lanes 1a–8a, corresponding to lanes 1–8, HDL incubation was omitted from the ligand blotting procedure. The unretained fraction, which was collected as a single pool, did not contain HDL-binding activity (not shown). Molecular weight markers are to the left of lane 1.

anti-rabbit immunoglobulins (Nordic, Tilburg, The Netherlands) and studied by fluorescence microscopy.

Results

Porcine liver proteins were solubilized in Triton X-100. The yield was about 2,600 mg of solubilized material from 100 g liver tissue (wet weight). This fraction was subjected to ion-exchange chromatography on DEAE-cellulose. Triton X-100 was then replaced by CHAPS to prevent interference in the ligand blot assay.29 Proteins were eluted with buffer containing CHAPS and NaCl. Samples of these fractions were analyzed for the presence of HDL-binding proteins in the ligand blot assay. A discontinuous NaCl gradient was used for elution. Two HDL-binding proteins of approximately 90 and 180 kDa molecular mass could be detected (Figure 1), eluting preferentially with NaCl concentrations between 50 and 125 mM NaCl. A third protein band of approximately 100 kDa was disregarded, as it was also observed in the ligand blot assay under control conditions (Figure 1, lanes 4a–6a).

The 50–125 mM NaCl eluate was pooled and subjected to Con A-Sepharose affinity chromatography. HDL-binding activity proved to be present in the retained fraction only (Figure 2), indicating that these HDL-binding proteins are glycoproteins. Apart from the 90- and 180-kDa proteins, two minor bands of approximately 110 and 130 kDa were observed. The presence of these bands was dependent on the preparation used. In some preparations, both were absent; in other preparations, only a 110-kDa HDL-binding protein was observed. In all cases, the 90- and 180-kDa proteins were by far the most prominent bands.

The retained fraction was analyzed in ligand blots using 125I-HDL in the absence or presence of a 20-fold excess (on a mole-per-mole basis) of unlabeled HDL or LDL (Figure 3). Again, two HDL-binding proteins of approximately 90 and 180 kDa were visible (Figure 3, lane A). The 110- and 130-kDa proteins were present as minor bands. Binding was completely inhibited when unlabeled HDL was present in the incubation medium (Figure 3, lane B), while LDL had little effect (Figure 3,
lane C). The use of either porcine or human HDL in the ligand blot assay resulted in an identical pattern of HDL-binding proteins in this fraction (Figure 4).

Further purification was obtained by repetitive preparative SDS-PAGE. The Con A-Sepharose retained fraction was subjected to SDS-PAGE. Slices containing proteins of 90 and 180 kd molecular mass, respectively, were cut out from the gels (Figure 5, left panel). Proteins were electroeluted from these gel slices and subjected to SDS-PAGE again. After two rounds of SDS-PAGE, gel slicing, and electrodialysis, the proteins were purified to homogeneity as judged from silver-stained gels. In a typical preparation, the yield from 2,600 mg solubilized protein was 480 μg and 260 μg of 90- and 180-kd protein, respectively. After protein transfer onto nitrocellulose, a small strip was subjected to the ligand blot assay. At the position of the HDL-binding proteins, a horizontal strip of the nitrocellulose sheet was cut out. Proteins were eluted from this strip with dimethyl sulfoxide and were used to immunize the rabbits. Two antisera were obtained, recognizing proteins of approximately 90 and 180 kd, respectively (Figure 5, right panel).

To further evaluate the specificity of the antisera obtained, two-dimensional gel electrophoresis was performed, and the results of immunoblotting and the HDL ligand blot assay were compared (Figure 6). The minute amounts of protein that must be detected after two-dimensional gel electrophoresis necessitate the use of the most sensitive detection method available (i.e., alkaline phosphatase–conjugated immunoglobulins as a secondary antibody). However, this results in an irrepressible background staining in the HDL ligand blot assay. On comparing the HDL-incubated blot (Figure 6a) with the control non–HDL-incubated blot (Figure 6b), spots representing HDL-binding proteins of 90 as well as 180 kd could be detected (Figure 6a), distinct from a number of other spots, most of which are nonspecific because they were also present in control incubations (Figure 6b). The 90- and 180-kd HDL-binding protein spots clearly correspond with the protein spots detected with the anti-HDL-binding protein antisera (Figures 6c and 6d). The HDL-binding proteins are apparently present in several isoforms, with different isoelectric points, presumably caused by differences in sugar moieties.

In immunoprecipitation studies with the antisera raised against the 180-kd HDL-binding protein, it was shown that with increasing amounts of antisera, HDL-binding activity to a 180-kd protein intensifies in the precipitate (Figure 7, upper panel), while it concomitantly attenuates in the nonprecipitated fraction (Figure 7, lower panel). No coprecipitation of the 90-kd HDL-binding protein was observed in these experiments. Thus, it can be concluded that the antisera obtained recognizes an HDL-binding protein of 180 kd. Attempts to obtain similar results using the antisera raised against the 90-kd HDL-binding protein were unsuccessful. This was due to the fact that no immunoprecipitation could be achieved with this antisera, as revealed by immunoblotting: no protein could be detected in the “precipitated” fraction, while the immunoreactivity in the nonprecipitated fraction remained unchanged (results not shown).

Immunohistochemistry was performed with these antibodies on semithin frozen sections of porcine liver.
FIGURE 7. Immunoprecipitation studies using the anti-180-kd high density lipoprotein (HDL)-binding protein antiserum. To 400 μg Con A-Sepharose eluate, various amounts of antiserum were added as indicated below. After incubation (16 hours, 4°C), 150 μl of a 1:1 suspension of protein A-Sepharose was added. After an additional incubation (2 hours, room temperature), the supernatant was separated from the immunoprecipitate by centrifugation (5 minutes, 400g). Both were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and analyzed in the ligand blot assay. Upper panel: Immunoprecipitate; lower panel: nonprecipitated fraction. Lanes 1–6 were incubated with 500, 200, 100, 50, 20, or 0 μg immunoglobulin G/ml, respectively. Molecular weight markers are to the left of lane 1.

The localization of the two HDL-binding proteins appeared to be markedly different (Figure 8). While the 90-kd HDL-binding protein is located within the hepatocellular plates, the 180-kd HDL-binding protein is present along the lining of the sinusoids.

Discussion

HDL is presumed to act as a vehicle for cholesterol in the reverse cholesterol transport pathway. In this pathway, one or more HDL-binding proteins could be involved.8,9 A variety of HDL-binding proteins has been described by several research groups using ligand blotting studies.14–19 Some groups detected two HDL-binding proteins within one tissue type.18,19 This diversity may in part be attributable to species and/or tissue specificity. In rat luteal cells, for instance, where HDL-cholesterol serves as a precursor for steroidogenesis, an HDL-binding protein of 58 kd has been reported.31 Recently, an 80-kd protein has been described in mouse adipose cells, which serve as a cholesterol depot.16 In many peripheral cells, a 110-kd HDL-binding protein is present,17 mediating cholesterol efflux from these cells by a nonendocytotic mechanism.20,21 On loading the cells with cholesterol, the amount of this 110-kd protein was shown to increase, suggesting a regulative mechanism for increased cholesterol efflux.17 Several groups have reported the presence of HDL-binding proteins in liver. However, their data are not consistent. In rat liver, HDL-binding proteins of 78 kd14 and 100 and 120 kd19 have been described, while in the rat hepatoma cell line

FIGURE 8. Immunohistochemistry of porcine liver sections. Semithin (0.5–1.0-μm) cryosections of porcine liver were incubated with polyclonal antibodies raised against the 90-kd (panel A) and the 180-kd (panel C) high density lipoprotein–binding proteins. Panels B and D are the corresponding phase-contrast images. Antigen–antibody complexes were visualized with fluorescein isothiocyanate–conjugated goat anti-rabbit immunoglobulins.
Fao, a 110-kd HDL-binding protein has been detected. In the human hepatoma cell line HepG2, both an 80-kd and a 110-kd HDL-binding protein have been reported. There are no reports on the functional differences between these proteins.

In this study, we show that in porcine liver, two major HDL-binding proteins of approximately 90 and 180 kd are present. These proteins were purified to apparent homogeneity and used to raise polyclonal antisera. Each of the antisera obtained recognized only one of the HDL-binding proteins, indicating that these proteins are unrelated, at least immunologically. Moreover, striking differences were observed in histochemical localization studies on porcine liver sections using these antisera. The distribution of the 90-kd HDL-binding protein within the hepatocellular plates is suggestive for a localization at or near the bile canaliculi, while the 180-kd HDL-binding protein is present along the lining of the sinusoids. These results suggest a functional difference between the two HDL-binding proteins described.

In the reverse cholesterol transport pathway, HDL delivers cholesterol to the liver for excretion into the bile. Recent evidence for this pathway has resulted from perfusion studies with monkey and rat liver. Therefore, a liver-specific HDL metabolism is obligatory. We describe two proteins in porcine liver that bind HDL in a ligand blot assay carried out on nitrocellulose filters. Their function in HDL metabolism remains to be elucidated. However, some speculations can be made regarding the differences in localization. Assuming that the 180-kd protein is a cell-surface protein, it could bind plasma HDL. In this respect, it could be equivalent to the peripheral HDL-binding protein of 110 kd that mediates cholesterol efflux. However, on binding to the 110-kd protein, no cellular uptake of HDL occurs. In contrast, HDL is taken up by liver cells. In hepatocytes, HDL can be converted to bile acids for excretion into the bile. However, a considerable part enters the bile as free cholesterol. The 90-kd HDL-binding protein, if indeed located near the bile canaliculi, is possibly interacting in this process. The 90-kd protein could also be a mediator for excretion of apolipoproteins A-I or A-II into the bile, where these apolipoproteins probably serve as cholesterol crystal formation inhibitors.

HDL particles can also be recrystallized or transported to lysosomes. In these routes, other still-undefined HDL-binding proteins could be involved. Indeed, we observed two minor HDL-binding protein bands after Con A-Sepharose chromatography in several preparations. One of these proteins has a molecular mass of 110 kd and might be identical to the 110-kd protein in HepG2 cells described by Graham and Oram. Further purification and characterization of these proteins is in progress.

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