EXPERIMENTAL BIOLOGY

HDL-Binding Lipoproteins on Human Fetal Hepatocytes

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Two types of binding sites for high-density lipoproteins (K_d=1 μg/ml and 20 μg/ml) were identified on human fetal hepatocytes and designated as P_1 and P_2. Ligand blotting has shown that P_1 site is protein with Mr 100 kD and P_2 site involves two proteins with Mr 105 and 110 kD. P_2 proteins were not detected when incubation with ligands was shortened from 24 h to 30 min. Molecular weight and activity of P_1 did not change considerably after reduction of disulfide bonds.

Key Words: *hepatocytes; primary culture; high-density lipoproteins; receptor*

It has been generally accepted [11] that high-density lipoproteins (HDL, d=1.125-1.216) extract excessive cholesterol (CL) from peripheral tissues and transport it to the liver. The mechanisms of CL extraction so far remain obscure. It was suggested that CL are removed from peripheral tissues by binding to some receptor-like structures located on cell surface [9]. Apolipoprotein A (apoA-I) provides high-affinity binding of HDL to these structures. Interaction of HDL with "receptors" ensures translocation of intracellular CL on the plasma membrane and its subsequent extraction by HDL [2,6].

HDL-binding proteins were identified in various cells and tissues [2,6,8]. Some proteins were partially purified, sequenced, and cloned. However, further experiments have shown that these HDL-binding structures probably are not involved in Cl transport. HDL-binding sites with high affinity and high association/dissociation rates were then discovered on the surface of some cell types [3,8]. Methods for the identification of these binding sites on rat hepatocytes have been developed at the Laboratory of Cell and Tissue Cultures (Cardiology Research-and-Production Center). These methods are based on the isotherms for ^{125}I-HDL and Scatchard plot analysis [1]. In the present study we describe two types of HDL-binding proteins that correspond to HDL-binding sites with K_d=1 mg/ml (P_1) and K_d=20 μg/ml (P_2) on the surface of human fetal hepatocytes.

MATERIALS AND METHODS

Hepatocytes were isolated from liver autopsy material obtained not later than 2-3 h after death.

Hepatocytes were isolated as described elsewhere [3]. A cannula was inserted into the left umbilical vein. The liver was first perfused with Ca^{2+} and Mg^{2+}-free Hanks' solution (400 ml) containing 5 mM EDTA at a rate of 20 ml/min then with the same solution without EDTA and then incubated

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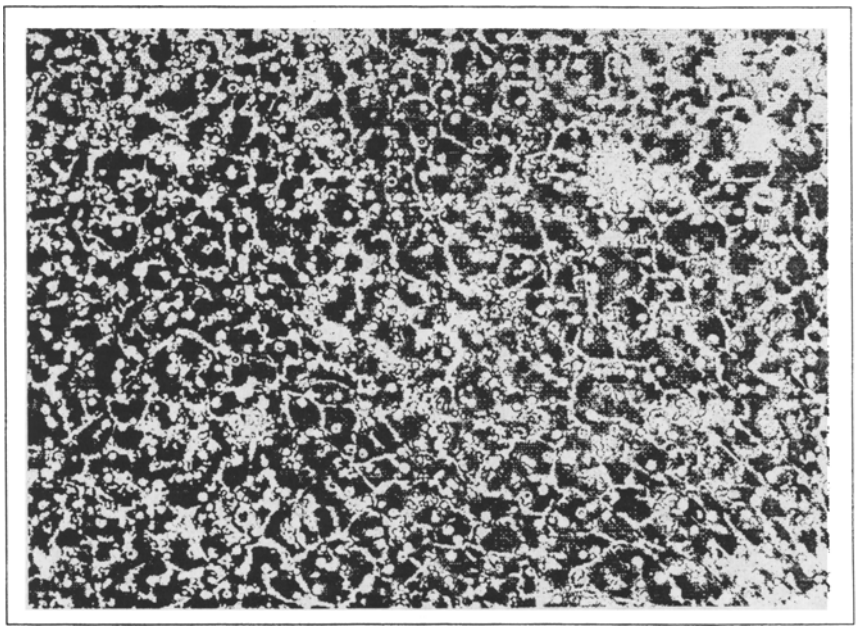

Fig. 1. Phase-contrast microscopy of human hepatocyte culture (×100).

with 1 mg/ml collagenase in Mg^{2+}-free Hanks' solution containing 5 mM $CaCl_2$ for 10-15 min. Hepatocytes were isolated from the resultant cell suspension by differential centrifugation (50*g*, 3 min 3 times) and subsequent centrifugation on 40% Percoll (250*g*, 10 min). The suspension contained 95% hepatocytes 90% of which were viable. The cells were seeded as described previously [3] and cultured in Williams' E medium (Sigma) containing 100 μg/ml kanamicyn (Sigma), 100 μg/ml fungizone (Flow Lab.), 100 nM dexamethasone, 100 nM estradiol, and 2 μg/ml insulin (Sigma). The medium was replaced every day. Primary cultures were used in experiments 48-96 h after seeding.

Isolation and radiolabeling of HDL and Scatchard analysis of HDL binding were performed as described previously [3]. The intracellular protein content was determined by the method [4] after alkaline hydrolysis of hepatocytes and neutralization with HCl.

To prepare the membrane fraction the hepatocytes were harvested into ice-cold stock solution (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 1 mM PMSF and 1 mM EDTA and homogenized at 24,000 rpm for 30 min (Ultra-Turrax T25, Janke and Kunkel IKA-Labortechnik). The homogenate was centrifuged at 250*g* for 10 min at 4°C. The supernatant that contained cytoplasmic membranes was centrifuged at 50,000g for 45 min at 4°C, pooled, frozen, and stored at -20°C.

The membranes were analyzed electrophoretically by the method [5] under denaturing and normal conditions (7.5% polyacrylamide gel). Electrical transfer on type BA85 nitrocellulose membranes (Schleicher and Schull) was carried out by the method [10]. The membranes were then cut into strips and blocked in the stock solution containing 5 mM $CaCl_2$ and 5% delipidated dry milk (blocking solution) at a room temperature for 1 h. Incubation with ligand (1-25 μg/ml HDL or low-density lipoproteins in blocking solution) was carried out at 4°C for 24 h and 30 min. The strips were washed from unbound ligand with 12 ml stock solution and fixed for 30 min in phosphate buffer containing 4% para-

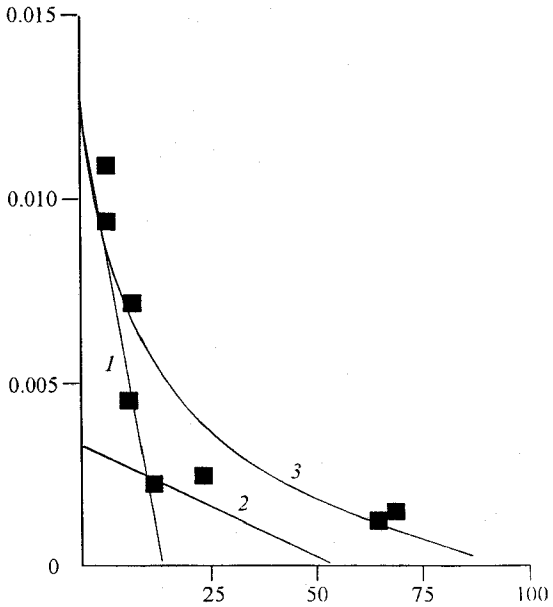

Fig. 2. Scatchard plots of specific binding. Abscissa: specifically bound ^{125}I-HDL_3, ng/mg cell protein; ordinate: bound-free ligand ratio. High-affinity *(1)* and low-affinity *(2)* components of specific linkage determined with the use of Ligand software; *3)* specific HDL binding. The values are the means of three parallel determinations in one out of five typical experiments.

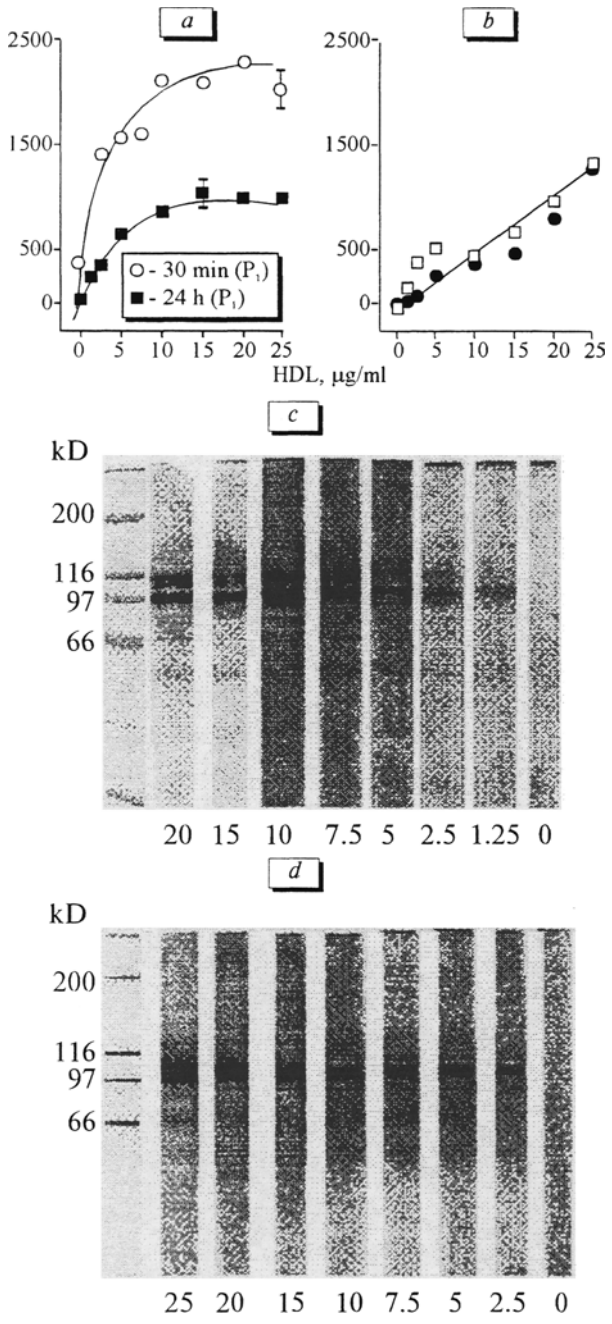

Fig. 3. Dose-dependent binding of HDL in ligand blotting. *a)* binding of HDL to P_1 binding sites; 24-h and 30-min incubation with ligand at 4°C; *b)* binding of HDL to P_2 binding sites. Ligand blotting after 24-h *(c)* and 30-min incubation *(d)* with ligand at 4°C. Abscissa: HDL concentration μg/ml; ordinate: intensity of band staining, arb. units (SigmaPlot, Sigma).

formaldehyde (pH 7.4). Unoccupied binding sites were blocked with the blocking solution for 15 min at room temperature. The HDL-receptors were identified with 1C5 monoclonal anti-human HDL antibody and anti-mouse IgG antibodies conjugated with alkaline phosphatase (Sigma). The reaction was developed at 4°C overnight.

RESULTS

Figure 1 is a phase-contrast microphotograph of a 48-h culture of hepatocytes isolated from human fetuses. The cells form a confluent monolayer, contain 1-2 nuclei and discernible nucleoli, form cell-to-cell contacts, and are firmly attached to the substrate. The hepatocytes remained viable and preserved their morphology for 96-144 h after seeding (the entire period of investigation).

High-affinity HDL-binding sites with Kd=2.5 μg/ml, k_{-1}=0.123 min^{-1}, and k_{+1}=0.041 min^{-1}/μg/ml were previously identified on the surface of some cell types [2,6,8], including rat hepatocytes [1]. The shortening of the period during which the ligand-receptor complexes were washed from unbound ligand is crucial for the identification of these binding sites. This approach was employed to reveal specific HDL-binding sites on human hepatocytes (Fig. 2). Two-component binding was observed after a single short-term washing with cold Hanks' solution. Scatchard plots with subsequent analysis of the curves by the method [7] revealed binding sites (P_1) with K_d=1 μg/ml (N=10 ng/mg cell protein) in addition to the previously revealed P_2 binding sites with K_d=20 μg/ml (N=50 ng/mg cell protein). This is consistent with our previous results obtained on rat hepatocytes. We have suggested that the same approach to the ligand blotting will allow us to identify the proteins corresponding to these binding sites.

Figure 3 illustrates dose-dependent binding of HDL to HDL-binding proteins in ligand blotting (electrophoresis under nondenaturing conditions). The protocol of ligand blotting was modified in comparison with methods [2,6,8]. First, ice-cold basal solution was used for a single washing the ligand-receptor complex from unbound ligand. This allowed us to identify ligand-receptor complexes described with high dissociation rates. The complexes were fixed under mild conditions: 4% paraformaldehyde in neutral phosphate buffer. Three HDL-binding proteins with Mr 97, 105, and 110 kD were revealed on nitrocellulose strips (Fig. 3, *c*). Only one protein (Mr 97 kD) was revealed after a short (30 min) incubation of strips with the ligand (fig. 3, *d*), suggesting that this protein (P_1) is a high-affinity component with K_d=1 μg/ml. Scanning of protein bands on the nitrocellulose filter shows that the binding of HDL with this proteins reaches a plateau (Fig. 3, *c, d*) at concentration which are an order of magnitude lower (Fig. 3, *a*), than that of the two other proteins (Fig. 3, *b*).

Further analysis of HDL binding in ligand blotting was carried out after electrophoretic separation of proteins under reducing conditions (Fig. 4, *a, b*).

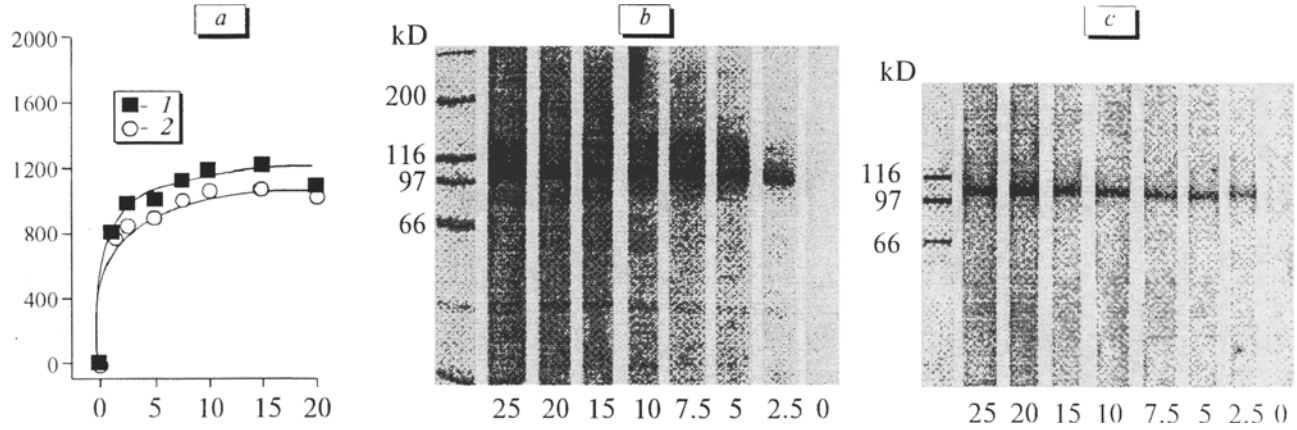

Fig. 4. Dose-dependent binding of HDL in ligand blotting (electrophoresis under reducing conditions). *a)* HDL binding to nitrocellulose strips during 24-h *(2)* and 30-min incubation *(1)* at 4°C. Ligand blotting during 24-h *(b)* and 30-min *(c)* incubation at 4°C.

HDL-binding protein with Mr 97 kD was identified after both 24-h and 30-min incubations. Two other proteins were not revealed. The parameters of HDL binding to this protein did not change considerably, since the binding reached a plateau at a concentration of 7.5 μg/ml as after electrophoresis under nonreducing conditions (Fig. 3, *c, d*) and in the presence of β-mercaptoethanol (Fig. 4, *b, c*). From these findings it was suggested that high-affinity HDL-binding protein (P_1) is a monomeric protein with Mr 97 kD and that its activity does not decrease after reduction o disulfide bonds.

There is a considerable body of evidence concerning the parameters of low-affinity HDL-binding proteins. The molecular weight of these proteins ranges from 66 to 120 kD [2,6,8] which hampers their comparison. However, the protein with Mr 100 kD, which was identified in the present study, is the only known HDL-binding protein with SS bonds (reduction of these bonds leads to a decrease in its electrophoretic mobility of this protein). HDL bound to this protein in the presence of Ca^{2+} (5 mM) and could not be identified when incubation was carried out in the blocking solution, although dry mild contains small amount of $CaCl_2$ (<1 mM). The protein identified in the present study is the only protein in which reduction of SS bonds does not lead to a decrease in the binding activity. It is noteworthy that this protein is present on human and rat hepatocytes. Thus, we have discovered and characterized a unique high-affinity HDL-binding protein which was previously detected by us and other researchers on different cell types [1,3,6,8].

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