Hepatic lipase is localized at the parenchymal cell microvilli in rat liver

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Hepatic lipase (HL) is thought to be located at the vascular endothelium in the liver. However, it has also been implicated in the binding and internalization of chylomicron remnants in the parenchymal cells. In view of this apparent discrepancy between localization and function, we re-investigated the localization of HL in rat liver using biochemical and immunohistochemical techniques. The binding of HL to endothelial cells was studied in primary cultures of rat liver endothelial cells. Endothelial cells bound HL in a saturable manner with high affinity. However, the binding capacity accounted for at most 1% of the total HL activity present in the whole liver. These results contrasted with earlier studies, in which non-parenchymal cell (NPC) preparations had been found to bind HL with a high capacity. To study HL binding to the different components of the NPC preparations, we separated endothelial cells, Kupffer cells and blebs by counterflow elutriation. Kupffer cells and endothelial cells showed a relatively low HL-binding capacity. In contrast, the blebs, representing parenchymal-cell-derived material, had a high HL-binding capacity (33 m-units/mg of protein) and accounted for more than 80% of the total HL binding in the NPC preparation. In contrast with endothelial and Kupffer cells, the HL-binding capacity of parenchymal cells could account for almost all the HL activity found in the whole liver. These data strongly suggest that HL binding occurs at parenchymal liver cells. To confirm this conclusion *in situ*, we studied HL localization by immunocytochemical techniques. Using immunofluorescence, we confirmed the sinusoidal localization of HL. Immunoelectron microscopy demonstrated that virtually all HL was located at the microvilli of parenchymal liver cells, with a minor amount at the endothelium. We conclude that, in rat liver, HL is localized at the microvilli of parenchymal cells.

INTRODUCTION

Hepatic lipase (HL) is present in the liver of most mammals, where it plays a central role in lipoprotein metabolism. Patients with HL deficiency have high levels of low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-triacylglycerols, circulating β-very-low-density lipoproteins and impaired clearance of chylomicron remnants, resulting in premature atherosclerosis [1]. The enzyme may be involved in the hepatic uptake of HDL-cholesterol [2]. Overexpression of HL in mice [3] and rabbits [4] leads to a decrease in plasma HDL-cholesterol levels. Exposure of HDLs to HL *in vitro* leads to an increased HDL-cholesterol uptake by rat hepatoma cells [5]. Marques-Vidal et al. [6] showed that HL is involved in the uptake of HDL-cholesterol esters in the perfused rat liver. The enzyme is also believed to play an important role in the catabolism of chylomicron remnants. When rats are depleted of HL, by injection of either heparin or antibody, the clearance of chylomicron remnants by the liver is impaired [7,8]. In addition, chylomicron remnants treated with HL are taken up more rapidly *in vivo* [9] and *in vitro* [10].

HL is synthesized in rat liver parenchymal cells and subsequently secreted into the extracellular space. It is generally accepted that HL (sometimes called hepatic endothelial lipase) is located at the luminal side of the liver endothelium similarly to lipoprotein lipase in extrahepatic tissues [11,12]. This localization is based on histochemical and biochemical evidence. Microscopical studies showed that HL is present in rat liver sinusoids [13,14]. Using electron microscopical techniques, Kuusi et al. [14] found HL at the luminal side of the endothelium. In addition, it was shown that HL binding to non-parenchymal liver cells (NPCs) was much higher than to parenchymal liver cells. The binding capacity of these cells *in vitro* would be sufficient to bind about 80% of the HL activity present in rat liver [15].

The localization of HL is of importance with regard to its proposed functions. Chylomicron remnant removal takes place in parenchymal cells, and an endothelial localization would exclude a direct role for HL in this process. Involvement of HL in the uptake of HDL-cholesterol (esters) would, with an endothelial localization, indicate that there exists a difference between the site of action of HL and the site of uptake of the HDL components. Therefore we reinvestigated the localization of HL in rat liver. Because the NPC fraction consists of endothelial cells, Kupffer cells and blebs, we studied HL binding to NPCs more closely and to isolated endothelial cells in particular. In addition, we carried out immunocytochemical studies at both the light-microscopic and electron-microscopic level.

MATERIALS AND METHODS

Animals

Male adult Wistar rats housed under controlled conditions were used. They were provided with normal rat chow an water *ad libitum*.

Liver endothelial cells in primary culture

A cell suspension was prepared from the liver of one rat as described by Seglen [16]. NPCs were centrifuged for 30 min at

Abbreviations used: HL, hepatic lipase; NPC, non-parenchymal cell; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FITC, fluorescein isothiocyanate; LRP, LDL-receptor-related protein; DiI, 3,3′-dioctadecylindocarbocyanine.

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800 ***g*** (4 °C) through a two-step density gradient of 50 % and 25 % isotonic Percoll (Pharmacia, Uppsala, Sweden) as described by Smedsrød and Pertoft [17]. The cells at the interphase were harvested, washed and seeded (0.5×10^6 cells/cm^2) on fibronectin-coated wells [17] in RPMI 1640 medium (supplemented with 2 mM L-glutamine, 200 μg/ml gentamicin and 50 μg/ml fungizone; all from Flow Laboratories, Irvine, Scotland, U.K.). After 2 h at 37 °C in an Heraeus incubator in a humidified atmosphere containing 5 % CO_2, the cultures were washed thoroughly with serum-free RPMI medium to remove non-adherent cells. Subsequently, the cultures were maintained for 20–22 h in medium supplemented with 20 % heat-inactivated newborn calf serum (Flow Laboratories).

Cells in suspension and in monolayer culture on coverslips were characterized by light and fluorescence microscopy (Leitz, Wetzlar, Germany). Endothelial cells were identified by their ability to accumulate 3,3′-dioctadecylindocarbocyanine (DiI)-labelled acetylated LDLs (BTI, Stoughton, MA, U.S.A.) [18]. Kupffer cells, which also accumulate DiI-labelled acetylated LDLs, were discriminated from endothelial cells by staining for endogenous peroxidase with diaminobenzidine (Sigma, St. Louis, MO, U.S.A.) as described [19]. Fat-storing cells were identified by the lack of DiI–acetylated LDL accumulation and by the presence in their cytoplasm of numerous lipid droplets displaying rapidly fading bluish autofluorescence under UV, characteristic of vitamin A [20]. Parenchymal cells, which are much larger than all other types of liver cells, were clearly recognized by light microscopy. At the time of the binding experiments, the monolayer cultures consisted of 85–90 % endothelial cells, 10–15 % fat-storing cells, less than 1 % Kupffer cells and no parenchymal cells.

HL binding to monolayer cultures of endothelial cells

Primary cultures of liver endothelial cells in 35 mm wells were used 22–24 h after seeding. Fibronectin-coated wells without cells were included in each experiment as a control. After five washes with PBS at 4 °C, the wells were incubated on ice for 2 h with different amounts of partially purified HL in 1 ml of PBS/1 % BSA. After the incubation, the wells were washed five times in PBS. The wells were scraped with a rubber 'policeman', and the cells were centrifuged and solubilized in 200 μl of PBS containing 4 mM CHAPS (Boehringer, Mannheim, Germany), 50 units/ml heparin, 1.5 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma), 1 mM EDTA, 1 mM benzamidine and 1 mM PMSF (Merck, Darmstadt, Germany). The samples were sonicated and the homogenates used for analysis of HL activity. DNA was measured fluorimetrically using 4′,6-diamidino-2-phenylindole (Boehringer) [21] and calf thymus DNA (BDH, Poole, Dorset, U.K.) as standard.

Isolation of HL

HL was isolated from heparin-containing rat liver perfusates [22]. Rat livers were flushed at 37 °C with Berry medium (140 mM NaCl, 5.4 mM KCl, 8.18 mM $MgSO_4$, 0.8 mM Na_2HPO_4, 25 mM $NaHCO_3$, 2.54 mM $CaCl_2$ and 6 mM D-glucose), followed by perfusion with Berry medium containing 10 % glycerol, 0.1 % BSA and 5 units/ml heparin. The heparin-containing perfusates were collected on ice, applied to a Sepharose–heparin column (1.5 cm × 15 cm; flow rate 36 ml/h) and washed with 3 bed vol. of 10 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl and 10 % glycerol. HL was eluted in the same phosphate buffer containing 0.1 % BSA, 10 % glycerol and 1 M NaCl. Triacylglycerol lipase activity was determined as described below and the peak fractions were pooled and stored at −80 °C. Specific activity of the HL used was measured using an ELISA [23] and was 29.0 m-units/μg.

Isolation of rat liver cell fractions

Rat liver cells were isolated by liver perfusion with collagenase as described by Seglen [16]. Parenchymal cells were isolated by centrifugation for 5 min at 50 ***g***. NPCs were collected by centrifugation of the supernatant at 400 ***g*** (10 min at 4 °C). The resuspended cell pellet was centrifuged through a 30 % Nycodenz gradient (Nycomed A/S, Oslo, Norway) to remove debris and erythrocytes. Endothelial cells, Kupffer cells and blebs were separated by counterflow elutriation at 3200 rev./min as described previously [24,25]. Blebs were eluted at a flow of 12 ml/min, endothelial cells at 25 ml/min and Kupffer cells at 70 ml/min. The different fractions were washed with Hanks buffer containing 0.3 % BSA (Hanks/BSA), centrifuged at 400 ***g*** (4 °C for 10 min) and resuspended in a small volume of Hanks/BSA.

HL binding to rat liver cell fractions

Cell fractions (about 100 μg of cell protein) were incubated with different amounts of HL in incubation buffer (20 mM Hepes, 5 mM KCl, 128 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 % BSA) for 10 min at 4 °C. After incubation, the cells were pelleted (10 min, 400 ***g***, 4 °C) and washed twice with incubation buffer without BSA. After resuspension of the cell pellet in 250 μl of PBS, HL activity was measured and protein content was determined by the method of Lowry et al. [26] with BSA as standard.

HL activity

HL activity was measured as triacylglycerol lipase activity in the presence of 0.6 M NaCl and 2.4 % BSA at pH 8.5. An artificial glycerol [9,10(n-^{3}H)]trioleate (Amersham International, Amersham, Bucks., U.K.) substrate in gum arabic was used [27]. Activity was expressed in m-units (nmol of non-esterified fatty acids released/min).

Immunocytochemistry

Rat livers were perfused as described [16]. The liver was flushed with Berry medium for 5 min at 37 °C. As a control, after the wash-out period, livers were perfused with 0.3 M NaCl as described by Schoonderwoerd et al. [23] or with heparin (5 units/ml) at 37 °C to remove HL. Thereafter, the liver was washed with Berry medium at 37 °C. To prevent internalization of HL, temperature was then lowered to 4 °C and the liver was perfused for 30 min in a recirculating manner with Berry medium containing anti-HL antibodies (0.3 mg of protein/ml) and 0.1 % BSA. The anti-HL antibodies we used were affinity-purified goat polyclonal immunoglobulins specific for rat HL as described previously [28], and 1 mg of antibody protein was able to inhibit 4000 m-units of enzyme. After a 5 min wash with Berry medium the tissue was fixed by perfusion with 150 ml of 2 % (w/v) paraformaldehyde in 0.2 M phosphate buffer, pH 7.4, excised and subsequently incubated in fixative for 2 h at 4 °C. For light-microscopic localization of HL, tissue blocks were embedded in Tissue Tek® and quick-frozen in liquid nitrogen. The blocks were stored at −80 °C. Cryostat sections (5 μm thick) were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat immunoglobulins (Nordic, Tilburg, The Netherlands; 1:80 dilution) and studied by fluorescence microscopy.

For immunoelectron microscopy, tissue blocks were stored in 2% paraformaldehyde/1 M sucrose in 0.1 M phosphate buffer. Immunoelectron microscopy on ultrathin frozen sections using rabbit anti-goat immunoglobulins conjugated with colloidal gold ($d = 10$ nm; Aurion, Wageningen, The Netherlands) was used to detect HL at the subcellular level [29].

RESULTS

Binding of HL to cultured endothelial cells

Overnight cultures of NPCs established in fibronectin-coated culture wells consisted of 85–90% endothelial cells, 10–15% fat-storing cells and virtually no Kupffer cells or hepatocytes. At 20–24 h after plating, most of the endothelial cells had spread. The wells contained 5.1 ± 1.5 μg of DNA/cm^2 (mean $\pm$ S.D., $n = 6$), which corresponds to 1.5×10^5 cells/cm^2 [30], similar to the figures reported by Smedsrød and Pertoft [17].

Binding to endothelial cells in culture was studied as described in the Materials and methods section. As shown in Figure 1, cell-associated HL activity increased with the concentration of HL added to the medium. Binding to empty fibronectin-coated wells was negligible. Binding appeared to be saturable; by Scatchard analysis (Figure 1, inset), the data were fitted to a single binding component with half-maximal binding at 71 ± 28 m-units/ml and a maximal binding capacity of 6.4 ± 2.9 m-units/mg of cell DNA (means $\pm$ S.D., $n = 3$). This corresponds to a binding of 50 m-units/10^9 cells, whereas NPCs in suspension are able to bind 5000 m-units/10^9 cells [15]. The low binding of HL to cultured endothelial cells raises doubts about the presumed localization of HL at the endothelium.

Binding of HL to rat liver cells

We have previously shown that preparations of NPCs bind HL activity with a high capacity [15]. This NPC fraction consists of several components (endothelial cells, Kupffer cells and blebs). We studied the binding of HL to these different NPC fractions. To this end endothelial cells, Kupffer cells and blebs were isolated from an NPC fraction by counterflow elutriation [24,25], and binding studies were carried out with these fractions. The

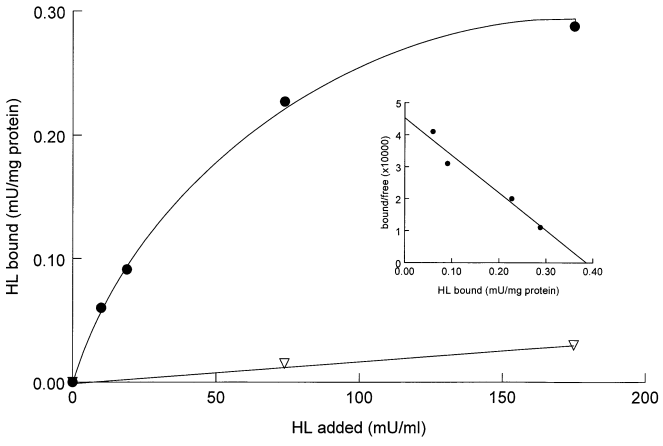

Figure 1 Concentration-dependent binding of HL to liver endothelial cells in primary culture

Cultures were incubated for 2 h on ice with different amounts of HL activity. Total binding to endothelial cells (●) and to empty fibronectin-coated wells (▽) is shown. Inset: Scatchard plot of total cell-associated binding. Data are representative of three similar experiments.

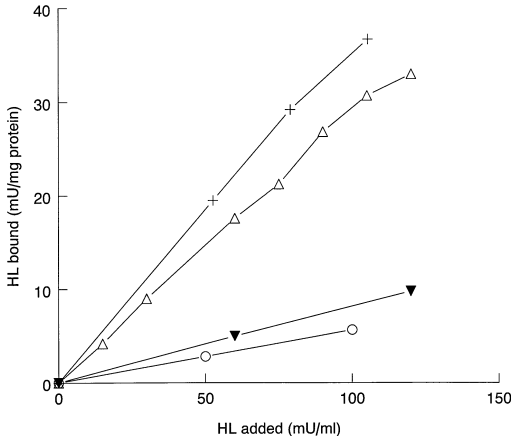

Figure 2 Concentration-dependent binding of HL to rat liver cells in suspension

Rat liver cell fractions after elutriation were incubated in suspension with different amounts of HL activity. After washing, cell-associated activity was measured. Binding to NPCs before elutriation (+) and to blebs (△), endothelial cells (○) and Kupffer cells (▼) after elutriation is shown. The data are representative of three independent experiments.

binding to the blebs was in the same range as for the whole NPC preparation (Figure 2) and started to show saturation at about 33 m-units/mg of protein. Half-maximal binding occurred at an HL concentration of about 65 m-units/ml (about 39 nM). Although no real saturation was observed, especially for the endothelial and Kupffer cells, binding capacities were calculated at a concentration of 100 m-units of HL/ml because *in vivo* the HL concentration will never exceed this value. Since about 54% of the total protein in the NPC fraction consists of blebs, it could be calculated that about 84% of all HL binding was attributable to the blebs (Table 1). In contrast endothelial and Kupffer cells bound less than 10 m-units/mg of cell protein and with a much lower affinity. The contribution of these cells to the binding of HL to the NPC preparation was less than 10% each (Table 1). Because blebs are of parenchymal origin, we also studied the binding of HL to freshly isolated parenchymal cells in suspension. Saturable binding with a maximum of about 4.5 m-units of HL activity/mg of protein was found (Figure 3). The affinity constant was estimated at an HL concentration of about 45 m-units/ml (27 nM). When calculated for the amount of parenchymal cell protein present in rat liver, the total binding capacity is 8000–10000 m-units (the HL activity present in rat liver is also 8000–10000 m-units). These results suggest that endothelial cells may contribute only marginally to the overall binding of HL to the liver, the HL-binding capacity of parenchymal cells being sufficient to account for all HL binding in whole rat liver.

Immunocytochemistry

We further studied the localization of HL by immunocytochemistry at the light microscopic and electron microscopic levels. First we visualized HL in rat liver by perfusion with polyclonal goat anti-(rat HL) antibodies followed by fixation because HL is very sensitive to aldehydes [31] and cannot be vizualized by classical techniques. Cryosections were stained with FITC-conjugated rabbit anti-goat IgGs as described in the Materials and methods section. A bright fluorescent signal was found in the sinusoids (Figure 4A). HL was exclusively found in the sinusoids; the larger vessels were not stained. All sections showed an even distribution throughout the tissue.

Table 1 Binding of HL activity to rat liver NPC fractions at an HL concentration of 100 m-units/ml

Values are means ± S.E.M. for three independent experiments.

	Binding (m-units/mg of protein)	Protein (%)	Percentage of total NPC binding
Blebs	33.6 ± 3.9	54.4 ± 7.7	84.0 ± 21.7
Endothelial cells	8.0 ± 4.2	15.7 ± 2.1	5.8 ± 3.8
Kupffer cells	8.5 ± 5.1	26.2 ± 4.9	10.2 ± 8.0

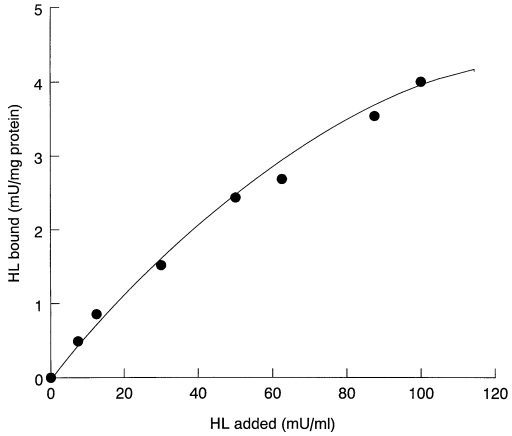

Figure 3 Concentration-dependent binding of HL to rat liver parenchymal cells in suspension

Data are representative of three independent experiments.

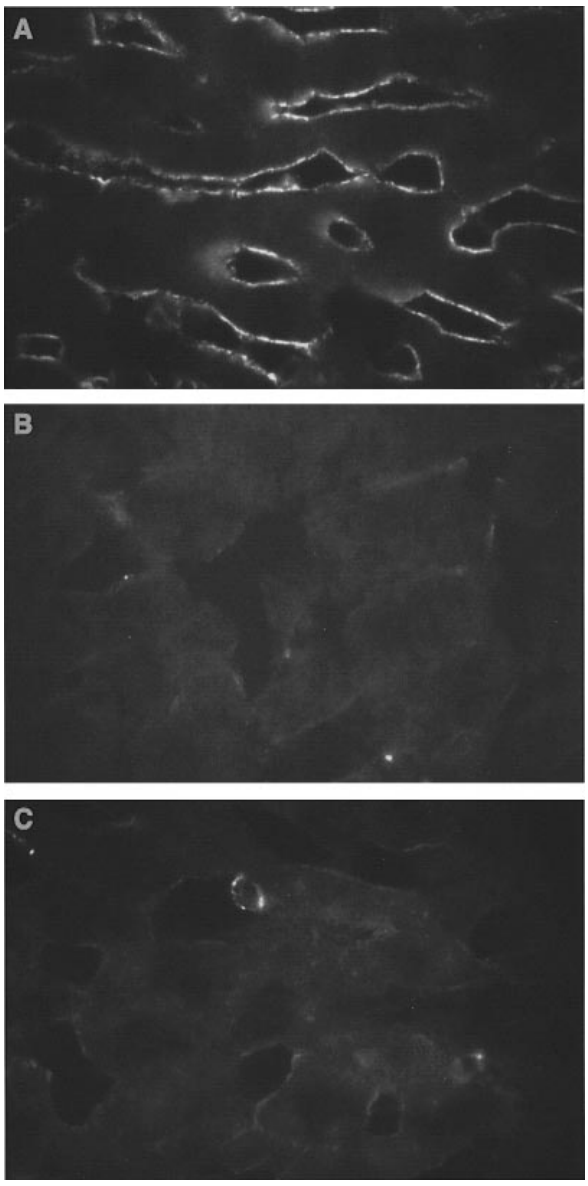

Figure 4 Fluorescence micrograph showing HL in rat liver

Livers were perfused with goat anti-HL antibodies and 5 μm cryosections were incubated with FITC-conjugated anti-goat IgGs. (**A**) A bright fluorescence in the sinusoids is seen which is evenly distributed over the cryosection (× 530). (**B**) After heparin preperfusion the signal has almost disappeared (× 530). (**C**) After preperfusion with 0.3 M NaCl the signal has again almost disappeared (× 530).

After perfusion of the livers with heparin, releasing more than 80 % of the HL activity, a very low fluorescent signal was found in the sinusoids (Figure 4B). Perfusion with 0.3 M NaCl, releasing about 60 % of the HL activity from the liver, also reduced the signal, but to a lesser extent than heparin (Figure 4C).

No conclusion about the subcellular localization of HL could, however, be obtained because of the insufficient resolution of fluorescence microscopy. The enzyme might be located at the parenchymal cells as well as at the endothelial cells. Therefore we performed immunoelectron microscopy using gold-labelled secondary antibodies to localize HL at the subcellular level. As shown in Figure 5 (top), HL was mainly present at the microvilli of the parenchymal cells in the Space of Disse. A minority of gold particles were present at the luminal side of the endothelial cells. When the rat liver was preperfused with heparin to release HL, the signal was reduced to background levels. In Figure 5 (bottom) a single immunogold particle at the luminal side of the endothelial cell is visible.

DISCUSSION

HL is thought to be localized at the endothelial cells in the liver, similarly to lipoprotein lipase in non-hepatic tissues. Recently, models have been proposed in which HL is directly involved in the binding to and internalization of lipoproteins in liver parenchymal cells [5,6,8–10,32]. An endothelial localization seems to be at variance with this function. However, it could be that only a minor part of HL, located at the parenchymal cells, acts as a lipoprotein-binding ligand. Therefore a spatial separation between a minor pool of HL involved in lipoprotein uptake and the major part of HL protein at the endothelial cells would exist. This would imply that the heparin-releasable HL does not represent the functional HL activity, but rather an endothelial lipase with another function. In this light, the exact localization of HL is of importance.

We previously showed that the binding of HL to rat liver NPCs was 20-fold higher than to parenchymal cells [15]. On the basis of these findings, we proposed that HL is localized at the liver endothelial cells. However, the NPC fraction consists not

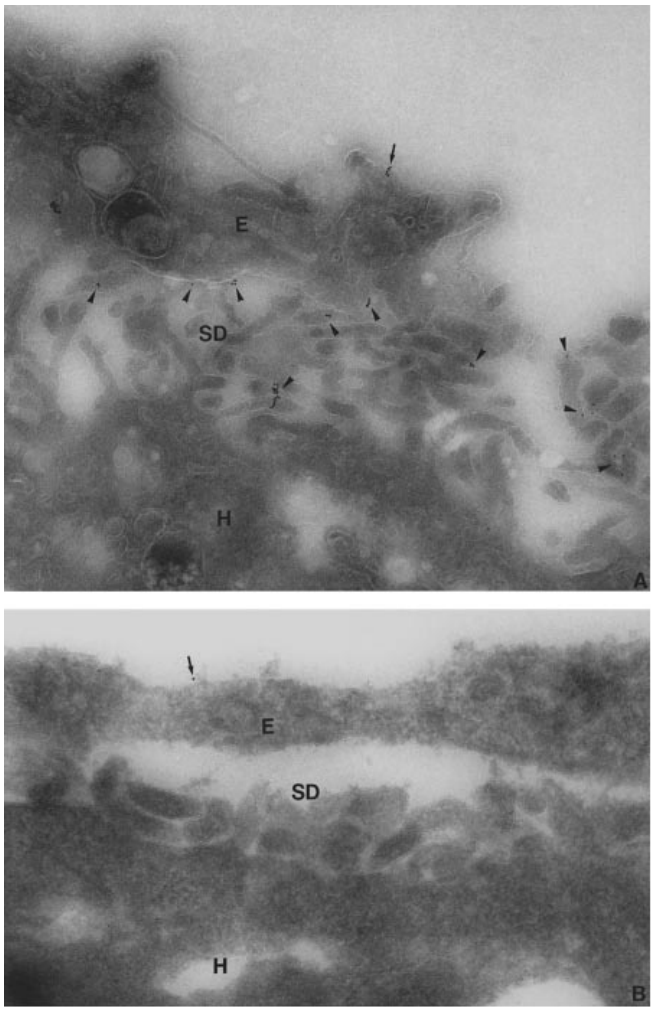

Figure 5 Electron-microscopical localization of HL in rat liver

HL was localized in rat liver after perfusion with goat anti-(rat HL) immunoglobulins. The anti-HL antibodies were visualized on 60 nm cryosections using rabbit anti-goat immunoglobulins conjugated with 10 nm colloidal gold. Top, the arrowheads show that HL is mainly localized in the Space of Disse (SD), at the microvilli of the hepatocytes (H). Some labelling of the luminal side of the endothelial cells (E) is present (arrow) ($\times$ 12750). Bottom, rat liver perfused with anti-HL antibodies after heparin perfusion. Virtually no immunogold particles are visible ($\times$ 14250).

only of endothelial cells, but also Kupffer cells and so-called blebs. These blebs are formed from parenchymal cells during cell isolation and have been characterized by Nagelkerke et al. [25]. When we studied the binding to the different elutriation fractions it was found that only about 7% of the binding to the NPC fraction could be attributed to the endothelial cells. The low binding capacity of freshly isolated endothelial cells could be due to loss of binding capacity during isolation. However, when the cells had recovered from the isolation procedure, binding of HL to the cultured cells was even lower. The reason for this is not clear. After 24 h culture, the cells are filled with numerous cytoplasmic vesicles not normally observed *in vivo*. This accumulation of probably endocytotic vesicles may suggest that transport in the cells is severely inhibited, and may cause intracellular sequestration of cell surface receptors such as those for HL. Another possibility is that HL binds to heparan sulphate proteoglycans which are exposed after isolation of endothelial cells. After culturing, the heparan sulphate proteoglycans are attached to the bottom of the plate and thus unable to bind HL.

Binding to freshly isolated Kupffer cells in suspension was low as well. In contrast, blebs present in the NPC fraction accounted for more than 80% of the binding to the NPC fraction. In addition, parenchymal cells in suspension bound 4–5 m-units/mg of protein which amounts to 8000–10000 m-units HL for the whole rat liver (the total enzyme activity present in a rat liver is 8000–10000 mU). The maximal binding for parenchymal cells (4–5 m-units/mg of protein) is much lower than for blebs (33 m-units/mg of protein) and thus it seems that the HL-binding site is specifically found in the blebs. However, the plasma membrane/volume ratio for blebs is four times higher than for parenchymal cells [25], and this partially explains the higher specific binding. The affinity constant was in the same range for parenchymal cells and blebs (27 and 39 nM respectively).

To study HL localization in rat liver further we used immunocytochemical techniques. Using immunofluorescence microscopy it was found that HL is present in the sinusoids, as shown previously [13]. The fluorescent signal was demonstrated to represent HL, because the signal disappeared on perfusion with heparin and also after perfusion with 0.3 M NaCl, which releases 60% of the HL activity [23]. Moreover, the signal was recovered when HL binding was restored by perfusion of the liver with purified HL (results not shown). However, we could not show the exact site of localization by light microscopy because the resolution was too low; therefore we used electron microscopy.

Electron-microscopical studies showed that *in situ* HL is mainly present at the luminal side of the parenchymal cells, thus confirming the *in vitro* studies. Some signal was found at the endothelium, but the parenchymal cell microvilli contained most of the gold particles. These particles appeared to be clustered. This is not due to a clustering of HL but is probably the result of the immunogold method. Slot and Geuze [33] showed that with the immunogold method the gold particles appear in small clusters because of the binding of two to three gold-labelled antibodies per antigen. Our findings are in contrast with those of Kuusi et al. [14], who found HL to be associated with the endothelium. However, in that study, the first and secondary antibodies were injected into the circulation at 37 °C. It may therefore be that the secondary antibody coupled to ferritin was taken up aspecifically by the endothelial cells. In our study, we perfused the liver with anti-HL antibodies at 4 °C and the gold-labelled antibodies were bound to the cryosections.

Therefore we conclude that HL is located at the microvilli of parenchymal cells in rat liver. This has implications for its functionality. HL plays a role in the processing of chylomicron remnants. When HL activity *in vivo* is abolished by either antibodies or heparin, chylomicron remnant clearance is impaired [7,8,10]. Because chylomicron remnants are mostly taken up by parenchymal liver cells it is of importance that HL is also present at this site to function either as a receptor or to process the remnant triacylglycerols.

HL was shown to be able to bind to LDL-receptor-related protein (LRP) in microtitre wells and the enzyme was internalized via LRP and subsequently degraded by HepG2 cells [34]. However, in liver, LRP is only present on parenchymal cells [35]. Therefore the localization of HL at parenchymal cells is in line with a presumed role of LRP in the degradation of HL.

Besides mediating chylomicron remnant uptake, HL is believed to play a role in reverse cholesterol transport by processing the phospholipids in HDLs and thus enabling a flux of cholesterol (esters) into parenchymal cells [2,5,6]. For this process, HL should also be in the vicinity of the parenchymal cell surface, otherwise the HDL components would have to be transported

from the sinusoidal space to the parenchymal cells. In conclusion, the localization of HL at the luminal side of the parenchymal cells is in line with its known functions.

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