Recognition of lactoferrin and aminopeptidase M-modified lactoferrin by the liver: involvement of proteoglycans and the remnant receptor

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1. Lactoferrin and aminopeptidase M-modified lactoferrin (APM-lactoferrin; which lacks its 14 N-terminal amino acids) inhibit the liver uptake of lipoprotein remnants. In the present study, the role of proteoglycans in the initial interaction of β-migrating very-low-density lipoprotein (β-VLDL), native and APM-lactoferrin with isolated rat parenchymal liver cells was investigated. Treatment of the cells with chondroitinase lowered the K_d of lactoferrin binding (from 10 to 2.4 μM), and the number of sites/cell (from 20×10^6 to 7×10^6), while heparinase treatment did not affect the binding. The binding characteristics of APM-lactoferrin and β-VLDL were not altered by treatment of the cells with chondroitinase or heparinase. It is concluded that proteoglycans are not involved in the initial binding of APM-lactoferrin and β-VLDL to parenchymal cells, while chondroitin sulphate proteoglycans are mainly responsible for the massive, low-affinity binding of native lactoferrin. 2. The binding of lactoferrin, APM-lactoferrin and β-VLDL to parenchymal liver cells was not influenced by the glutathione S-transferase-receptor-associated protein (GST-RAP) ($97.2 \pm 4.0\,\%$, $95.5 \pm 3.7\,\%$ and $98.5\,\%$ of the control binding), while the binding of α_2-macroglobulin was fully blocked at 10 μg/ml GST-RAP ($1.8 \pm 0.5\,\%$ of the control binding). Since GST-RAP blocks the binding of all the known ligands to the low-density lipoprotein (LDL)-receptor-related protein (LRP), it is concluded that LRP is not the initial primary recognition site for lactoferrin, APM-lactoferrin and β-VLDL on parenchymal liver cells. 3. We showed earlier that APM-lactoferrin, as compared with lactoferrin, is a more effective inhibitor of the liver uptake of lipoprotein remnants ($49.4 \pm 4.0\,\%$ versus $80.8 \pm 4.8\,\%$ of the control at 500 μg/ml respectively). We found in the present study that β-VLDL is able to inhibit the binding of APM-lactoferrin to parenchymal liver cells significantly ($74.9 \pm 3.3\,\%$ of the control; $P < 0.002$), while the lactoferrin binding was unaffected. It is concluded that a still unidentified specific recognition site (the putative remnant receptor) is responsible for the initial binding of remnants to parenchymal cells and it is suggested that the partial cross-competition between APM-lactoferrin and β-VLDL may be of further help in the elucidation of the molecular nature of this recognition site.

INTRODUCTION

Lactoferrin is an iron-binding glycoprotein belonging to the transferrins, which is able to bind two Fe^{3+} ions with high affinity [1]. It consists of a single, bilobed polypeptide chain with a relative molecular mass of 76500 to which two glycans are attached through N-glycosidic linkages [2–4]. The protein is synthesized by neutrophils and glandular epithelial cells [5,6]. The physiological role of lactoferrin is still unclear, but it has been suggested that lactoferrin plays a protective role during bacterial infection [7,8] and may prevent iron-induced lipid peroxidation [9].

When injected intravenously, lactoferrin is rapidly removed from the circulation of rats, mice and rabbits [10–15]. The rapid clearance was found to be mainly due to association of lactoferrin to the liver. After injection of lactoferrin into rats a rapid association with the parenchymal liver cells occurs, followed by a relatively slow internalization. *In vitro* studies, with freshly isolated rat liver parenchymal cells, showed that lactoferrin binds to these cells with low affinity (K_d about 10 μM) but high capacity (20×10^6 sites/cell) [15].

In a recent study, we enzymically removed the 14 N-terminal amino acids of lactoferrin using aminopeptidase M [16]. The resulting aminopeptidase M-modified lactoferrin (APM-lactoferrin) was also rapidly cleared from the circulation by parenchymal liver cells. It was, however, internalized much more rapidly by these cells than native lactoferrin. A further striking difference was the significantly altered binding characteristics. Compared with native lactoferrin, APM-lactoferrin was found to bind to parenchymal liver cells with much higher affinity (K_d 187 nM) to a much lower number of binding sites (approx. 750000 sites/cell). The 14 N-terminal amino acids that are removed from lactoferrin by aminopeptidase M contain a 4-arginine cluster of lactoferrin at positions 2–5 [17]. These results thus suggest that these residues may be involved in the massive, low-affinity association of lactoferrin with the liver. A possible explanation for the massive interaction of native lactoferrin with the liver may be found in binding to the extracellular matrix, especially proteoglycans.

An important feature of lactoferrin and APM-lactoferrin is their ability to inhibit the association of apolipoprotein E (apoE)-bearing lipoproteins to parenchymal liver cells [16,18,19]. APM-lactoferrin was found to be an even more potent inhibitor than native lactoferrin. Arginine residues of lactoferrin and APM-lactoferrin are crucial for recognition by parenchymal liver cells and their capacity to inhibit hepatic uptake of apoE-bearing lipoproteins [15,16,20]. The site in APM-lactoferrin that mediates its high-affinity binding to parenchymal liver cells and is re-

Abbreviations used: apoE, apolipoprotein E; APM-lactoferrin, aminopeptidase M-modified lactoferrin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; GST-RAP, glutathione S-transferase-receptor-associated protein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; Lp(A), Lipoprotein(a); LRP, LDL-receptor-related protein; β-VLDL, β-migrating very-low-density lipoprotein.

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sponsible for blocking the binding of apoE-bearing lipoproteins to the remnant receptor is, however, not yet identified. Based on its amino acid sequence the Arg/Lys-enriched sequence at positions 25–31 [17,21], which resembles the receptor binding site of apoE to the low-density-lipoprotein (LDL) receptor [22], is a good candidate.

The receptor responsible for the hepatic uptake of apoE-bearing lipoproteins, such as β-migrating very-low-density lipoprotein (β-VLDL) and chylomicron remnants, is, tentatively, called the remnant receptor. The molecular nature of this receptor is under intensive investigation, but as yet the receptor has not been definitively identified. Several distinct receptors are considered to be involved in the liver uptake of remnants. Among the LDL receptor gene family, the LDL receptor and the LDL-receptor-related protein (LRP) are suggested candidates. The LDL receptor is known to bind apoE and is estimated to account for about half the removal of remnants in humans [23]. The LRP was initially proposed by Herz et al. to serve as apoE-specific lipoprotein remnant receptor [24]. It is a multifunctional receptor that mediates uptake of α_2-macroglobulin, plasminogen activator–inhibitor complexes, bovine lactoferrin and lipoprotein lipase (LPL) [25–28]. LRP also binds β-VLDL, but only if it is additionally enriched with apoE. Several groups claim that it may function as an uptake system for apoE-rich lipoproteins [29–34]. Prolonged blockade with glutathione S-transferase-receptor-associated protein (GST-RAP) for 5 days in LDL receptor knockout mice led to an increase in circulating chylomicron and β-VLDL remnants [35]. Another candidate protein, the lipolysis-stimulated receptor (LSR), is a receptor that is activated by free fatty acids and has a high affinity for triacylglycerol-rich lipoproteins [36,37].

Recent publications from Mahley and co-workers [38,39] suggest a role for proteoglycans in the initial interaction of lipoprotein remnants with the liver. Proteoglycans are proteins that have one or more attached glycosamidoglycan chains, with highly negatively charged sulphate (e.g. chondroitin- and heparan sulphate) and carboxylate groups [40]. A large number of ligands are known to bind to proteoglycan structures, these include apoE [41], LPL [42], LPL complexes with β-VLDL, LDL, Lipoprotein(a) [Lp(a)] [43–45] and bovine lactoferrin [38]. It is suggested that binding of remnants to proteoglycans forms an essential initial step for subsequent LRP-mediated uptake [39,46].

Since the uptake of apoE-containing lipoproteins can be completely blocked by APM-lactoferrin these proteins were used to provide more insight in to the binding characteristics of lipoprotein remnant recognition. In the present study, we characterized the interaction of native and APM-lactoferrin with isolated rat parenchymal liver cells, in relation to the potential interaction sites for lipoprotein remnants. In particular, the role of proteoglycans and the involvement of LRP in the binding of (APM)-lactoferrin and β-VLDL to these cells were studied.

EXPERIMENTAL

Chemicals

Human lactoferrin (iron-saturated) was obtained from Serva, Heidelberg, Germany. Aminopeptidase M (amino acid aryl amidase EC 3.4.11.2) was purchased from Boehringer Mannheim, Germany. Fucoidin, collagenase (type IV), BSA (fraction V), heparinase (type I) and chondroitin lyase ABC were from Sigma, St. Louis, MO, U.S.A. Dulbecco's modified Eagle medium (DMEM) was from Gibco, Irvine, Scotland. ^{125}I (carrier free) in NaOH and ^{35}S as sulphate were from Amersham International, Amersham, Bucks., U.K. All other chemicals were of analytical grade.

Preparation of APM-lactoferrin

Modification of lactoferrin with aminopeptidase M was done as described in detail previously [16]. In brief, lactoferrin (15 mg) was incubated at 37 °C with 1.5 mg of aminopeptidase M in 1.5 ml of PBS (8 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl) supplemented with 0.15 mM $CoCl_2$. The reaction was followed by monitoring the release of acid-soluble UV-absorbing material from lactoferrin. After completion of the reaction, the solution was concentrated by ultrafiltration (Centricon 30, Amicon, Beverly, MA, U.S.A.), and APM-lactoferrin was separated from the enzyme by FPLC using a Superose 12 column (50 cm × 1.6 cm; Pharmacia, Uppsala, Sweden). The column was eluted with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl and 0.01 M EDTA at a flow rate of 6 ml/h.

Isolation of β-VLDL

Male Wistar rats (200–220 g) were maintained for 14 days on a cholesterol-rich chow that contained 2 % (w/v) cholesterol, 5 % (v/v) olive oil and 0.5 % cholic acid (Hope Farms, Woerden, The Netherlands). The rats were starved 24 h before blood was collected via the abdominal aorta. The sera of the rats were pooled and β-VLDL was isolated as described earlier [20].

Labelling of (APM)-lactoferrin and β-VLDL

Lactoferrin, APM-lactoferrin and β-VLDL were radioiodinated at pH 10.0 with carrier-free ^{125}I according to a modification [47] of the ICl method [48]. Free ^{125}I was removed by Sephadex G50 gel filtration, followed by dialysis against PBS for 20 h at 4 °C, with repeated changes of buffer. β-VLDL was dialysed as described above against PBS supplemented with 10 μM EDTA.

Isolation of parenchymal liver cells

Male Wistar rats (250–325 g) were anaesthetized and parenchymal liver cells were isolated by perfusion of the liver with 0.05 % collagenase at 37 °C by the method of Seglen [49], modified as described previously [50]. The obtained parenchymal cells ($\geqslant$ 95 % viable as judged by 0.2 % Trypan Blue staining and $\geqslant$ 99 % pure) were resuspended in DMEM supplemented with 2 % (w/v) BSA (pH 7.4).

Culture of parenchymal liver cells

Parenchymal liver cells were isolated as decribed above. Cells were seeded on 12-well cluster plates at a density of 0.5×10^6, and were maintained at 37 °C in a 5 % CO_2/95 % air atmosphere in Williams' E medium supplemented with 10 % (v/v) heat-inactivated bovine calf serum, 2 mM L-glutamine, 20 m-units/ml insulin, 100 units/ml penicillin and 100 units/ml streptomycin. The medium was renewed at 4 h after seeding. Experiments were performed with cells that had been cultured for 18–20 h.

Treatment of cells with heparinase I and chondroitin lyase ABC

Isolated rat parenchymal liver cells (approx. 16 mg of cell protein) were incubated for 40 min at 37 °C in the presence of heparinase I (2.4 units/ml [51]) or chondroitin lyase ABC (0.24 unit/ml [52]) in DMEM supplemented with 2 % (w/v) BSA (pH 7.4) in a total volume of 4 ml. Control cells were incubated without enzyme. After incubation the cells were centrifuged at 50 ***g*** for 1 min at 4 °C and washed once with DMEM containing 2 % (w/v) BSA (pH 7.4). The cells were subsequently used for binding studies.

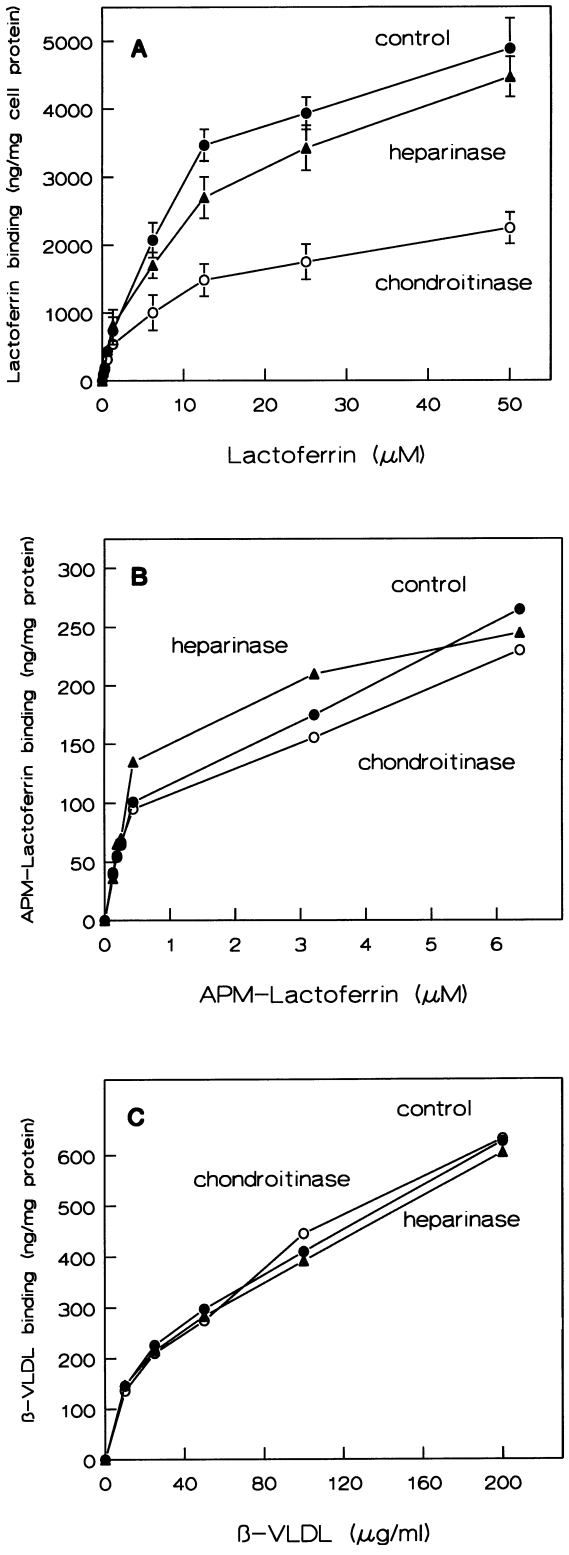

Figure 1 Effect of treatment of isolated rat parenchymal cells with heparinase I or chondroitin lyase ABC on the binding of APM-lactoferrin and β-VLDL

Freshly isolated parenchymal liver cells were incubated with heparinase I (2.4 units/ml; ▲) or chondroitin lyase ABC (0.24 unit/ml; ○) for 40 min at 37 °C. Control cells were incubated without enzyme (●). After washing, the cells were incubated with ^{125}I-lactoferrin (**A**), ^{125}I-APM-lactoferrin (**B**), or ^{125}I-β-VLDL (**C**) for 2 h at 4 °C at the indicated concentrations. Binding is expressed as ng of ligand/mg of cell protein. Values represent means (±S.E.M.) of 2–3 experiments.

Binding studies with parenchymal liver cells

Aliquots of 0.5 ml of the cell suspensions (containing 1–2 mg of cell protein) were incubated with radiolabelled ligands and competitors at indicated concentrations. Incubations were carried out in plastic containers (8.5 ml; Kartell) for 2 h at 4 °C under continuous shaking (150 rev./min). The incubations with cultured cells were performed in 12-well plates and the incubation volumes were adjusted to 0.7 ml with Williams' E medium containing 2% (w/v) BSA. After incubation, the cells were washed three times with wash buffer (50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.2% BSA) followed by a wash step with wash buffer without BSA. Finally, cells were lysed in 1 ml of 0.1 M NaOH and their radioactivity and protein content were determined.

RESULTS

Effect of treatment of freshly isolated rat parenchymal cells with heparinase I and chondroitin lyase ABC on the binding of (APM)-lactoferrin and β-VLDL

Parenchymal cells were enzymically treated with heparinase (type I; 2.4 units/ml) or chondroitin lyase ABC (chondroitinase; 0.24 unit/ml), enzymes which digest heparan sulphate and chondroitin sulphate respectively. After incubation of the cells with the above-mentioned enzymes, the viability of the enzyme-treated cells did not differ significantly from that of control cells.

To demonstrate that heparinase and chondroitinase treatment resulted in removal of the glucosaminoglycan sulphates we labelled freshly isolated parenchymal liver cells with [^{35}S]sulphate for 2 h at 37 °C. Heparinase or chondroitinase and a combination of both enzymes released 31.6±3.4, 28.4±2.1 and 52.1±3.7% of the radioactivity respectively.

Enzyme-treated and control cells were incubated at 4 °C with various concentrations of ^{125}I-lactoferrin, ^{125}I-APM-lactoferrin and ^{125}I-β-VLDL in order to determine the binding characteristics of the ligands to the cells. Treatment of the cells with chondroitinase (0.24 unit/ml), resulted in changed binding parameters for lactoferrin (Figure 1A). Binding parameters were determined by using non-linear regression, including an aspecific component (Graphpad, ISI software, San Diego, CA, U.S.A.). The K_d value was lowered from 10 to 2.5 μM by chondroitinase treatment and the amount of binding sites/cell from 20×10^6 to 7×10^6 binding sites/cell. Treatment of cells with higher concentrations of chondroitinase (up to 1.2 units/ml) did not have an additional effect on the binding of lactoferrin. The binding of lactoferrin was not significantly affected by treatment of the cells with heparinase I at a concentration of 2.4 units/ml. Under similar conditions, LPL-mediated LDL binding to isolated parenchymal cells was reduced by approx. 50% [53], and binding to HepG2 cells was reduced by 85% [43]. Treatment of the cells with heparinase or chondroitinase did not influence the binding of APM-lactoferrin and β-VLDL (Figures 1B and 1C). Also the binding of lactosylated BSA was not influenced (results not shown). The characteristics of binding of all three ligands to the control cells were very similar to those obtained in our earlier studies using cells not digested with heparinase and chondroitinase [15,16,54].

Effect of GST-RAP on the binding of (APM)-lactoferrin, β-VLDL and α_2-macroglobulin to freshly isolated rat parenchymal cells

To investigate the possible involvement of the LRP/α_2-macroglobulin receptor in the binding of (APM)-lactoferrin, β-VLDL and α_2-macroglobulin to parenchymal liver cells, we

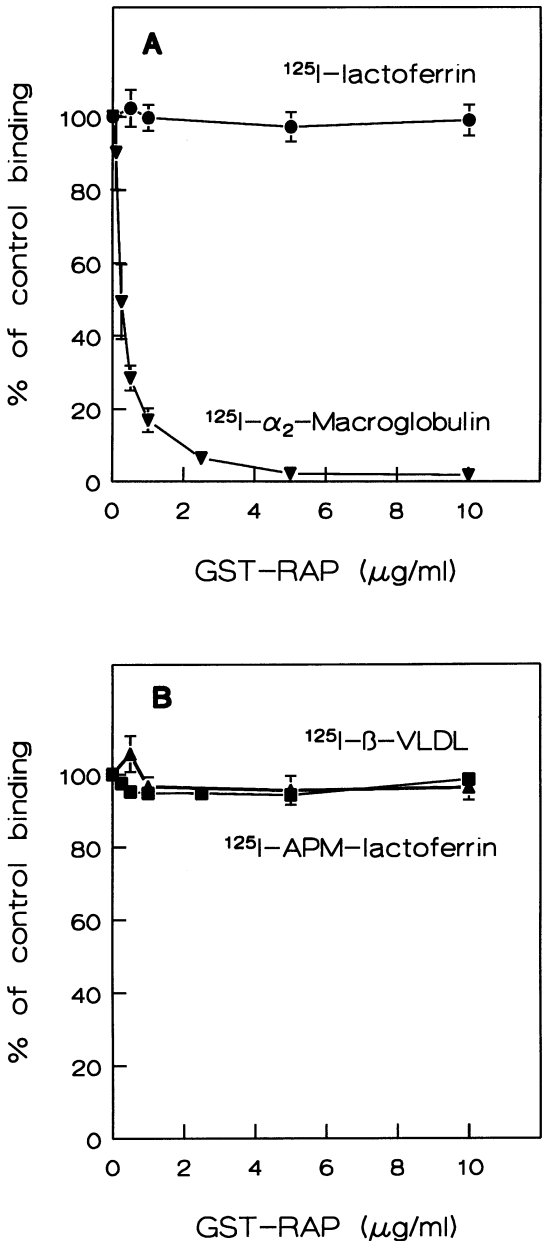

Figure 2 Effect of GST-RAP on the binding of lactoferrin, APM-lactoferrin and α_2-macroglobulin to isolated parenchymal liver cells

Freshly isolated parenchymal cells were incubated with ^{125}I-lactoferrin (0.13 μM; ●), α_2-macroglobulin (3.6 nM; ▼) (**A**), ^{125}I-APM-lactoferrin (0.13 μM; ■), or β-VLDL (10 μg/ml; ▲) (**B**) for 2 h at 4 °C in the presence of the indicated concentrations of GST-RAP. The binding is expressed as percentage of the binding of ^{125}I-lactoferrin (109 ± 5 ng/mg of cell protein), ^{125}I-α_2-macroglobulin (34.5 ± 4.9 ng/mg of cell protein), ^{125}I-APM-lactoferrin (22 ± 0.5 ng/mg of cell protein) or ^{125}I-β-VLDL (144 ± 5 ng/mg of cell protein) in the absence of GST-RAP. Values are means ± individual variation of two experiments.

studied the effects of increasing concentrations of the GST-RAP on the binding of these proteins (Figure 2). GST-RAP is a small soluble protein with a relative molecular mass of 39000, which was found to co-purify with LRP [55]. This protein inhibits LRP-mediated uptake of all known ligands including apoE-enriched lipoproteins as well as α_2-macroglobulin [25,29]. The binding of native lactoferrin and APM-lactoferrin to parenchymal liver cells was not influenced by addition of GST-RAP in concentrations

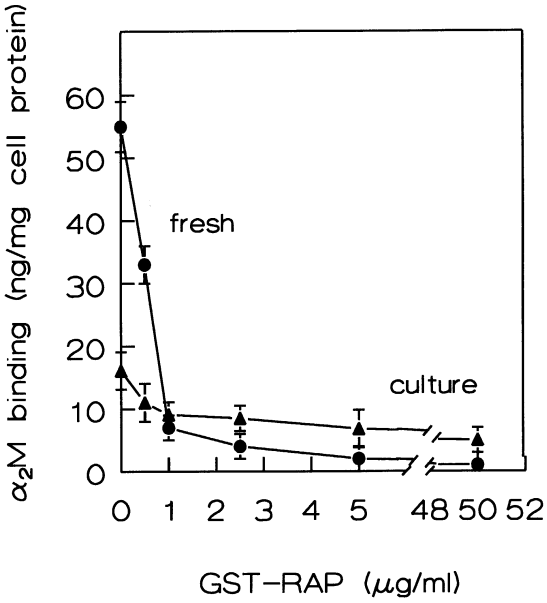

Figure 3 Effect of GST-RAP on the binding of α_2-macroglobulin (α_2-M) to cultured and freshly isolated parenchymal liver cells

Cultured parenchymal liver cells were incubated with ^{125}I-α_2-macroglobulin (3.6 nM) for 2 h at 4 °C in the presence of the indicated concentrations of GST-RAP (▲). The binding is expressed as ng/mg of cell protein. As a control the data with freshly isolated parenchymal liver cells are indicated (●). Values are means ± individual variation of two experiments.

up to 10 μg/ml (97.2 ± 4.0 % and 95.5 ± 3.7 % of the control binding, respectively). Also the binding of β-VLDL was not influenced by addition of GST-RAP (98.5 % of the control binding), whereas binding of α_2-macroglobulin was fully blocked at this concentration (1.8 ± 0.5 % of the control binding).

Effect of GST-RAP on the binding of α_2-macroglobulin to cultured parenchymal liver cells

To investigate the possibility that the inability of GST-RAP to block the interaction of β-VLDL with liver cells is caused by the obligatory collagenase isolation of liver cells, we cultured the cells for 18–20 h, so that the surface properties may be restored. However, during culture, the binding of ^{125}I-α_2-macroglobulin decreased significantly from 55.0 ± 3.7 ng/mg of cell protein with freshly isolated cells to 16.3 ± 3.2 ng/mg of cell protein after 18 h of culture (Figure 3). Although GST-RAP blocked the binding of α_2-macroglobulin to freshly isolated cells completely (97.7 % inhibition at 50 μg/ml GST-RAP), the binding of α_2-macroglobulin to cultured cells was only inhibited by 67 % by GST-RAP. These results indicate that the expression of LRP on the cell membrane is actually lost with cells in culture.

The binding values for (APM)-lactoferrin and β-VLDL, and the absence of an effect of GST-RAP on the binding of these ligands to cultured rat parenchymal liver cells, were not significantly different from the data with freshly isolated cells (results not shown).

Effect of β-VLDL on the binding of lactoferrin and APM-lactoferrin

In previous studies it was reported that lactoferrin and APM-lactoferrin do inhibit the uptake of lipoprotein-remnants, such as β-VLDL, by parenchymal liver cells, both *in vivo* and *in vitro*

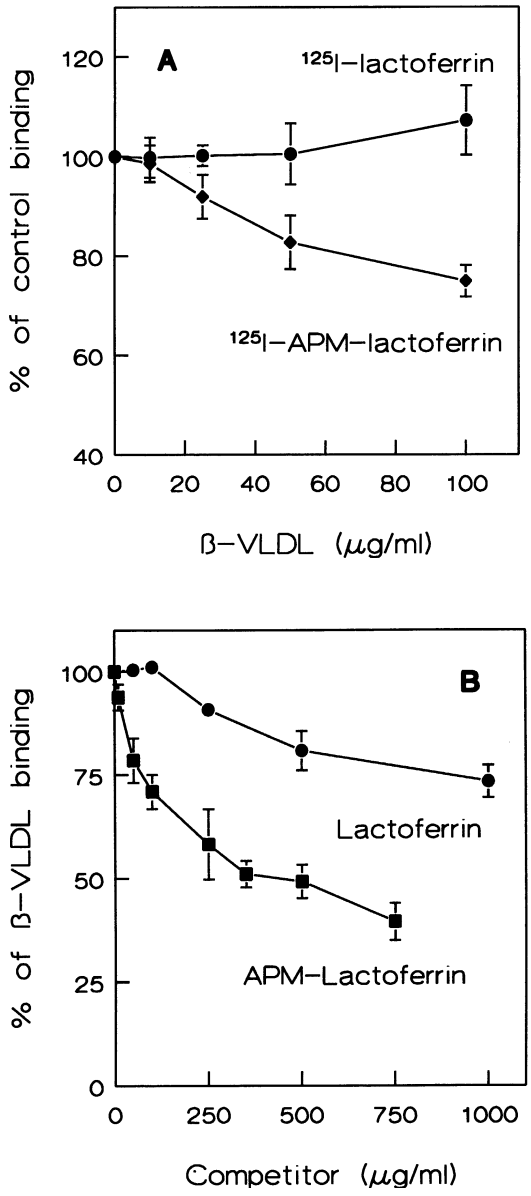

Figure 4 Effect of β-VLDL on the binding of lactoferrin and APM-lactoferrin to isolated parenchymal liver cells and vice versa

Freshly isolated parenchymal cells were incubated with ^{125}I-lactoferrin (●) or ^{125}I-APM-lactoferrin (■) at a concentration of 0.13 μM for 2 h at 4 °C in the presence of the indicated concentrations of unlabelled β-VLDL (**A**). The binding is expressed as percentage of the binding of ^{125}I-lactoferrin (109 ± 5 ng/mg of cell protein) and ^{125}I-APM-lactoferrin (22 ± 0.5 ng/mg of cell protein) in the absence of β-VLDL. Values are means ± S.E.M. of three experiments. (**B**) shows the effect of unlabelled lactoferrin (●) and APM-lactoferrin (■) on the binding of ^{125}I-labelled β-VLDL to rat parenchymal liver cells [16].

[16,18–20]. In the present study, we investigated the effect of β-VLDL on the binding (at 4 °C) of radiolabelled (APM)-lactoferrin (Figure 4A). The binding of APM-lactoferrin was significantly inhibited by β-VLDL. At a concentration of 100 μg of β-VLDL/ml, the binding of APM-lactoferrin decreased to 74.9 ± 3.2 % of the control binding ($P < 0.002$). Addition of β-VLDL to the incubation medium in concentrations up to 100 μg/ml had no effect on the binding of native lactoferrin (107.2 ± 6.9 % of the control binding at 100 μg/ml). In the converse experiment APM-lactoferrin was shown to be a more potent inhibitor (50 % inhibition of the β-VLDL binding with 0.5 mg/ml APM-lactoferrin) than native lactoferrin (50 % inhibition of the control binding with 4 mg/ml) [16] (Figure 4B).

DISCUSSION

In the present study, we investigated the role of proteoglycans and LRP as potential recognition sites in the initial interaction of lipoprotein remnants with the rat parenchymal liver cells.

Proteoglycans are constituents of the extracellular matrix, possessing highly charged sulphate (like chondroitin sulphate and heparan sulphate) and carboxylate groups. A large number of ligands are known to bind to proteoglycans including apoE, LPL complexes and β-VLDL enriched with apoE [41–45]. Treatment of cells with heparinase (type I) and chondroitin lyase ABC (chondroitinase) led to digestion of heparan sulphate and chondroitin sulphate residues respectively. The binding of APM-lactoferrin to liver parenchymal cells was not affected by prior treatment of the cells with heparinase or chondroitinase. The binding of lactoferrin to parenchymal cells, however, is markedly altered by treatment of the cells with chondroitinase. The dissociation constant was lowered from 10 to 2.4 μM and the amount of binding sites/cell was reduced from 20×10^6 to 7×10^6 binding sites/cell. Treatment of the cells with heparinase did not affect the binding characteristics of lactoferrin. Our present data thus indicate that the high-capacity, low-affinity binding of lactoferrin to freshly isolated rat parenchymal liver cells is largely due to binding to chondroitin sulphate groups of proteoglycans (for about 65 %). Probably, the binding is the result of an electrostatic interaction between the negatively charged chondroitin sulphate groups and the positively charged 4-arginine cluster of native lactoferrin (residues 2–5), which is absent in APM-lactoferrin. The binding of lactoferrin to chondroitinase-treated cells, however, remains higher than the binding of APM-lactoferrin to parenchymal liver cells (750000 binding sites/cell). Although chondroitin sulphate is thus of major importance, additional sites may interact with the cluster of 4-arginine residues of lactoferrin.

Our findings differ from recent data of Ji and Mahley [38], in which the interaction of bovine lactoferrin with heparan sulphate proteoglycans on HepG2 and Chinese hamster ovary (CHO) cells is described. The reason for this difference may be related to the cells studied (HepG2 and CHO cells versus freshly isolated rat parenchymal liver cells) as various cell types express different proteoglycans [56]. In addition, Ji and Mahley used bovine lactoferrin in these studies [38]. The structure of bovine lactoferrin differs at essential sites from that of human lactoferrin, i.e. in bovine lactoferrin the cluster of four arginine residues at the N-terminus is absent. This structure is, however, as presently shown, essential for the binding of human lactoferrin to chondroitin sulphate proteoglycans.

To determine the role of proteoglycans in lipoprotein remnant recognition, we also studied the effects of heparinase and chondroitinase on the binding of β-VLDL to parenchymal liver cells. Our results indicate that, in contrast to β-VLDL enriched in apoE [39] and complexes of β-VLDL with LPL [43], the interaction of native β-VLDL with parenchymal liver cells is not affected by treatment of the cells with heparinase or chondroitinase, and thus not mediated by heparan or chondroitin sulphate proteoglycans. The interaction of β-VLDL + apoE and LPL complexes with β-VLDL, LDL and Lp(a) with several types of cells [39,43–45] is significantly reduced by pretreatment of these cells with heparinase, a finding also confirmed for freshly isolated parenchymal liver cells in similar experiments in our laboratory [53].

To investigate the potential role of the LRP/α_2-macroglobulin receptor in the binding of (APM)-lactoferrin, β-VLDL and α_2-macroglobulin to parenchymal liver cells, the effects of GST-RAP on the binding of these proteins were studied. This fusion protein inhibits LRP-mediated uptake of apoE-enriched lipoproteins as well as the α_2-macroglobulin interaction with cultured cells [25,29]. The binding of both native and APM-lactoferrin by parenchymal liver cells was not influenced by addition of GST-RAP (97.2 ± 4.0 and $95.5 \pm 3.7\%$ of the control binding respectively), whereas the binding of α_2-macroglobulin was completely blocked ($1.8 \pm 0.5\%$ of the control binding). Earlier, Willnow et al. reported that the degradation of bovine lactoferrin could be inhibited by RAP in FH fibroblasts lacking the LDL receptor [27]. Furthermore, Willnow et al. reported in a recent paper that high concentrations of GST-RAP (up to 200 μg/ml) in mice resulted in a retarded plasma clearance of α_2-macroglobulin and an accumulation of remnant-like particles in the circulation at 5 days after treatment. High concentrations of GST-RAP (50–200 μg/ml) are, however, also able to inhibit the interaction of lipoproteins with the LDL receptor significantly [57,58], while the initial interaction of chylomicron remnants with the liver is only slightly affected. From our study it thus becomes clear that LRP and the LDL receptor do not play a significant role in the initial binding of (APM)-lactoferrin and β-VLDL to parenchymal liver cells, while it is still possible that LRP might be involved in the subsequent internalization.

In previous studies it was shown that lactoferrin blocks the recognition of lipoprotein remnants, including β-VLDL, by the liver parenchymal cells, both *in vivo* and *in vitro* [16,18–20]. Because lactoferrin also interacts with proteoglycans, these findings appear to be consistent with proteoglycan-mediated remnant binding. However, as found recently [16], APM-lactoferrin is even more effective in blocking the interaction of lipoprotein remnants with the liver, while its interaction with the parenchymal cells is not affected by treatment of the cells with heparinase or chondroitinase. The present data indicate that β-VLDL, at a concentration of 100 μg/ml, is able to reduce the binding of APM-lactoferrin significantly to $74.9 \pm 3.2\%$ of the control value. So for the first time it is shown that a lipoprotein remnant can, at least partially, inhibit the interaction of a protein (APM-lactoferrin) with liver cells.

As reported earlier the maximal amount of lipoprotein remnants that can be bound to rat liver parenchymal cells is estimated to be approx. 9000 [20]. As the amount of binding sites for APM-lactoferrin is much higher (750000 sites/cell) one has to assume that the remnant receptor is not the only receptor system involved in the recognition of APM-lactoferrin or that the relatively large remnants bind to multiple receptor sites. This might explain the relatively high concentrations of APM-lactoferrin (250–500 μg/ml per 350–700 nM) needed to compete for the binding of ^{125}I-β-VLDL, while the K_d value for APM-lactoferrin binding is 200 nM.

In conclusion, our data indicate that chondroitin sulphate proteoglycans do play an important role in the interaction of lactoferrin with the liver. APM-lactoferrin and β-VLDL, however, do not bind to proteoglycans. Furthermore, the LRP appears to be of minor importance in the initial binding of (APM)-lactoferrin and β-VLDL to rat parenchymal liver cells, while β-VLDL competes partly for the APM-lactoferrin binding. Earlier studies showed that APM-lactoferrin is an effective inhibitor of the interaction of native β-VLDL with parenchymal cells [16]. It may be suggested that, in addition to binding to the remnant receptor, APM-lactoferrin still interacts with additional recognition sites. However, the presently indicated cross-competition between β-VLDL and APM-lactoferrin may indicate that APM-lactoferrin can be a further aid in elucidating the nature of the site which is responsible for the initial recognition of remnants by the liver.

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