Zonal Distribution of Chylomicron Remnant Uptake in Rat Liver Parenchymal Cells

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Abstract. The binding and internalization of chylomicron remnants in rat hepatocytes originating from the periportal and perivenous zones was compared. The hepatocyte sub-populations were separated by centrifugal elutriation and incubated with ^{125}I-labelled chylomicron remnants at 37 °C (to measure binding and internalization) or 4 °C (to measure initial binding). Periportal and perivenous cells bound and internalised similar amounts of remnants up to a concentration of about 25 μg remnant protein per assay, but at higher concentrations the periportal cells were able to internalise significantly more remnants. When excess unlabelled low density lipoprotein was added to the incubations, little effect on the kinetics of either binding or internalization of the remnants was observed. Lactoferrin, an inhibitor of uptake via the remnant receptor, also did not affect the initial binding of the remnants to either cell type, but decreased internalization to similar extents in both sub-populations. These results suggest that periportal hepatocytes have a greater capacity for the uptake of chylomicron remnants than perivenous cells, and that the remnant receptor plays a more important role than the low density lipoprotein receptor in both sub-populations. This acinar heterogeneity parallels that reported previously for cholesterol *de novo* synthesis, bile formation, lipid content and hepatic lipase secretion.

Key words: Chylomicron remnant binding — Chylomicron remnant uptake — Dietary cholesterol — Hepatic zonation — Parenchymal cells specialization.

Abbreviations: ApoE, apolipoprotein E; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoproteins; LRP, LDL receptor-related protein; LSR, lipolysis stimulated receptor; PBS, phosphate buffe-

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red saline; PP, periportal; PV, perivenous; β-VLDL, β-migrating very low density lipoproteins.

Introduction

It has been known for some time that the heterogeneity of parenchymal liver cells is an important factor in the regulation of many liver functions (Kietzmann and Jungermann 1996). Cells in the periportal zone are exposed to blood rich in oxygen, substrates, hormones, etc., while those in the perivenous zone are exposed to blood depleted in these substances, and the two sub-populations have been shown to differ in both their subcellular structures and enzyme complements (Kietzmann and Jungermann 1996).

The liver has a central role in the maintenance of cholesterol homeostasis, as it is responsible for the uptake of cholesterol from the circulating lipoproteins and its subsequent excretion from the body via the bile, and is also a major site for the synthesis and secretion of cholesterol into plasma in new lipoproteins. A number of processes involved in hepatic cholesterol metabolism have been found to be heterogenously distributed in the periportal and perivenous liver parenchyma. In normal physiological conditions, 3-hydroxyl-3-methylglutaryl Co-enzyme A reductase, the enzyme regulating *de novo* cholesterol synthesis (Rudney and Sexton 1986), is believed to be located mainly in the periportal zone (Singer et al. 1984; Li et al. 1988). Capacity for bile formation also appears to be greater in periportal cells (Jones et al. 1978; Daoust 1979), though the activity of cholesterol 7α hydroxylase, the rate-limiting step for bile acid synthesis (Botham 1986), has been reported to be approximately 8 fold higher in perivenous as compared to periportal hepatocytes (Ugele et al. 1991). These apparently contradictory observations can be explained by greater feedback inhibition of bile acid synthesis by bile acids returning to the liver in the enterohepatic circulation (Vlahcevic et al. 1991) in the periportal zone, where the hepatocytes would be exposed to higher blood bile acid levels (Botham 1986).

Although the removal of lipoproteins carrying lipids of both dietary and endogenous origin is a major function of the liver in cholesterol metabolism, information about the zonation of the processes involved is scarce. Chylomicron remnants transport cholesterol from the diet to the liver, where they are rapidly taken up from the circulation after their formation from chylomicrons by the action of endothelial cell-bound lipoprotein lipase (Redgrave 1983). It is likely, therefore, that there is a concentration gradient in blood levels of remnants, similar to that postulated for bile acids (Botham 1986), between the periportal and perivenous parenchyma. The precise mechanism whereby the liver removes chylomicron remnants is still a matter of debate (for a review see Cooper 1997). Chylomicron remnant uptake requires apolipoprotein E (apo E), which is present on the surface of the particles and is

also secreted by hepatocytes and bound to the outside of the membrane (Brown et al 1991) The process is believed to be mediated by the low density lipoprotein (LDL) receptor (Nagata et al 1988, Choi et al 1991) and also by a second endocytic receptor (Ishibashi et al 1994, van Berkel et al 1995), the lipoprotein-remnant receptor, which is likely to be the LDL receptor-related protein (LRP) (Brown et al , 1991, Willnow et al 1992, Mokuno et al 1994) or perhaps the lipolysis stimulated receptor (LSR) (Mann et al 1995) Recent evidence from studies with a variety of experimental models including the LDL receptor knockout mouse, however, suggests that initial binding of chylomicron remnants to hepatocytes occurs at sites separate from the LDL receptor and the LRP, possibly involving glycosaminoglycan-bound hepatic lipase and apoE, with the two receptors subsequently taking part in the endocytosis of the particles (Herz et al 1995, de Faria et al 1996, Krapp et al 1996)

Separation of liver parenchymal cells into sub-populations originating from the periportal and perivenous regions has been used to study zonal differences in a wide variety of liver functions (Kietzmann and Jungermann 1996) Using a digitonin-pulse perfusion method, Vooschuur et al (1994) have found that the binding of β migrating very low density lipoprotein (β-VLDL) to cell membranes was similar in both periportal and perivenous cells β-VLDL are a mixture of cholesteryl ester rich chylomicron remnants and VLDL remnants, which accumulate in the serum of cholesterol fed animals or patients with type III hyperlipoproteinemia (Fainaru et al 1988), and are thought to be recognised by the lipoprotein-remnant receptor in rat liver (Harkes et al 1989) In the present work, we have investigated the binding and internalization of chylomicron remnants by isolated rat liver periportal (PP) and perivenous (PV) parenchymal cells, using remnants prepared in rats *in vivo* and radiolabelled with ^{125}I In addition, the involvement of lipoprotein receptors in these processes was studied using specific inhibitory ligands

Materials and Methods

Animals and materials

Male Sprague Dawley rats (*Rattus norveticus ab alba*) (300 350 g) were used for chylomicron and chylomicron remnant preparation, and hepatocyte donors weighed 220–250 g Rats were kept on a standard low-fat pellet diet, allowed access to food and water *ad libitum*, and housed under constant day length (12 h) and temperature (25 °C) ^{125}I (specific activity 15 5 mCi/μg) was purchased from Amersham International plc (Bucks, UK) Total cholesterol and triacylglycerols were measured using assay kits from Menarini (Florence, Italy) and Sigma Chemical (St Louis, USA), respectively Collagenase was purchased from Boehringer Mannheim GmbH (Mannheim, Germany) Dulbecco's modified Eagle's medium (DMEM), gelatin

bovine serum albumin (BSA), modified Lowry reagent, heparin, human lactoferrin and all other chemicals were from Sigma Chemical (St. Louis, USA).

Preparation and radioiodination of chylomicron remnants

Lymph chylomicrons and circulant chylomicron remnants were obtained basically according to Lambert et al. (1996). Rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) 1h after being given 1 ml of corn oil supplemented with α-tocopheryl acetate (6.71 IU of vitamin E/ml) by stomach tube. The thoracic duct was then cannulated and the chyle was collected for 16–18 h in the presence of ampicillin (0.1 mg/ml). Large chylomicrons (> 100 nm in diameter) were isolated by ultracentrifugation ($6 \times 10^5 \times g$ for 1 min in a fixed-angle rotor at 12°C) after layering the chyle under NaCl ($d = 1.006$ g/ml). For the preparation of chylomicron remnants, rats were anaesthetized as above and functionally hepatectomized by ligation of all major blood vessels supplying the liver and the gut. Chylomicrons containing approximately 30 μmol triacylglycerol and 50 mg of added glucose were then injected into the left ileolumbar vein. 45 min later, the blood was withdrawn from the abdominal aorta with simultaneous infusion of 1% BSA in 0.9% NaCl via the right ileolumbar vein. The serum obtained was layered under NaCl ($d = 1.006$ g/ml) and ultracentrifuged at $6 \times 10^7 \times g$ for 1 min at 12°C. The chylomicron remnants harvested by aspiration of the top 1–1.5 ml fraction were purified by a further centrifugation step at $d = 1.006$ g/ml for $3.2 \times 10^7 \times g$ for 1 min at 12°C.

Chylomicron remnants were radiolabelled with ^{125}I using iodine monochloride as described by Contreras et al. (1983). About 90% of the total radioactivity in the ^{125}I remnants was precipitated by 10% trichloracetic acid, and about 30% was found associated with lipids after exhaustive extraction with chloroform: methanol (2:1, v:v). The specific radioactivity was 11–40,000 cpm/μg protein.

Preparation of LDL

Human LDL was isolated by ultracentrifugation (Vieira et al. 1996) of fresh plasma obtained from fasted normolipemic subjects (aged 22–28). Briefly, plasma was adjusted to a density of 1.21 g/ml with solid KBr, and 3 ml was layered under 2.3 volume of 20 mmol/l potassium phosphate buffer, pH 7.4, containing 150 mmol/l NaCl and 1 mmol/l EDTA in ultracrimp polyallomer tubes. Tubes were centrifuged at 48,000 rpm for 3 h at 15°C in TFT 55.38 rotor. Bands containing LDL were harvested by tube slicing and dialysed extensively at 4°C against 150 mmol/l NaCl, 0.5 mmol/l EDTA, pH 7.4. Preparations were filtered (0.22 μm) and used within 2 weeks of their isolation.

Preparation and characterization of periportal and perivenous hepatocytes

Liver parenchymal cells were isolated by the two-step collagenase perfusion method of Seglen (1976). Cells were washed twice in Krebs-Henseleit buffer containing 20

mmol/l glucose and 2 5 mmol/l $CaCl_2$, and once in the so-called incubation medium consisting of DMEM supplemented with 20 mmol/l glucose, 1% BSA, 10 mmol/l pyruvate, 10 mmol/l HEPES and 44 mmol/l sodium bicarbonate The pellet was suspended in the incubation medium and filtered once through a 200 μm-pore mesh and twice through a 75 μm-pore nylon gauze Hepatocytes were fractionated using a Beckman JE6-B elutriator rotor on a Beckman J2-21 centrifuge adapted for operation at low speed Routinely 120×10^6 cells suspended in 6 ml of the incubation medium were submitted to centrifugal elutriation PP and PV cells were eluted from the rotor, using Krebs-Henseleit buffer supplemented with 1 mmol/l $CaCl_2$, 5 mmol/l glucose, 10 mmol/l pyruvate, 6 5 mmol/l HEPES and 0 05% gelatin (elutriation buffer), by changes in rotor speed and buffer flow rate Four populations of cells were recovered at 950 rpm-18 ml/min, 950 rpm-21 ml/min (PP cells), 950 rpm-24 ml/min and 880 rpm-24 ml/min (PV cells) Cells were counted and sized using a Coulter counter (Multisizer II) with a 70 μm diameter aperture Cell viability, as determined by Trypan Blue exclusion, always exceeded 85% in the final hepatocyte suspension Alanine and tyrosine aminotransferase activities were measured with a commercial assay Kit (Sigma Chemical) and according to the method of Granner and Tomkins (1970), respectively The size, cytoplasmic complexity, mitochondria content and ploidy of the PP and PV cells was determined in a flow cytometer (EPICS, model 752, Coulter, Hialeh, FL) equiped with an argon laser, using conventional fluorescent dyes (Gumucio et al 1981, Sultan et al 1989)

Cell binding and internalization of ^{125}I chylomicron remnants

Periportal and perivenous hepatocytes ($1\ 5 \times 10^6$ cells in 1 5 ml) were pre-incubated in the incubation medium supplemented with 1% BSA at 37°C for 2 h to restore the capacity of hepatocytes to take up chylomicron remnants (Sultan et al 1989) The incubations were carried out in glass siliconized vials with continuous gassing with 95%O_2 5% CO_2 After this time, ^{125}I chylomicron remnants were added and the incubations were continued under similar conditions at 37°C For experiments at 4°C the vials were transferred to an ice bath and pre-incubated for 10 min prior to addition of the remnants When required, lactoferrin or unlabelled LDL were added at this stage The cells were then separated from the medium by centrifugation ($1000 \times g$, 3 min), washed with phosphate buffered saline (PBS) pH 7 4 containing 2 mg/ml BSA (1 ml) three times and with PBS only (1 ml) once Cell pellets were resuspended in PBS containing heparin (5 mg/ml, 150 units/mg) and incubated at 4°C in a shaking bath After 1 h, the cells were separated from the medium by centrifugation ($1000 \times g$, 3 min), washed with PBS (1 ml) and treated with 1 mol/l NaOH (1 ml) Radioactivity in the cell and medium samples was determined using a Packard Tri-Carb gamma counter

Other analytical methods

Total cholesterol and triacylglycerols in lipoproteins were enzymatically determined with assays from Menarini and Boehringer Mannheim, respectively. Protein concentrations in cells were measured as described by Bradford (1976) and in lipoproteins as described by Peterson (1977), with BSA as a standard.

Results and Discussion

Periportal and perivenous parenchymal cells were characterized according to the distribution pattern of a number of marker enzymes and cell parameters which have been shown to reflect the asymmetry. The estimated PP/PV ratio of alanine aminotransferase activity (1.44) and tyrosine aminotransferase activity (1.15) were in close agreement with published values (Sumner et al. 1983; Tosh et al. 1988; Vooschuur et al. 1994) as were the size (PP, 19 μm; PV, 22 μm), cytoplasmic complexity (PP/PV ratio of 0.6), mitochondria content (PP/PV ratio of 1.4), and ploidy (higher in PV than in PP) (Gumucio et al. 1981; Sumner et al. 1983; Tosh et al. 1988; Vooschuur et al. 1994).

Initial experiments demonstrated that the binding of radioactivity from ^{125}I chylomicron remnants at 37 °C (defined as that released by heparin) reached a maximum after incubation with the cells for 30 min, while initial binding at 4 °C (defined as the total associated with the cells) reached a maximum after approximately 120 min. These incubation times, therefore, were chosen for subsequent experiments. Binding and internalization (defined as the radioactivity remaining associated with the cells after treatment with heparin) after incubation of rat periportal and perivenous hepatocytes with ^{125}I chylomicron remnants for 30 min at 37 °C are shown in Figure 1. At concentrations of remnants up to approximately 25 μg/assay (12.5 μg/ml), binding and internalization was similar in the two types of cells. At higher concentrations, however, both parameters decreased in the perivenous hepatocytes. In contrast, when the cells were incubated with the ^{125}I chylomicron remnants for 2 h at 4 °C, initial binding was similar in periportal and perivenous hepatocytes over the range of concentrations used (Fig. 2). These results suggest that periportal parenchymal cells have a higher capacity for the uptake of chylomicron remnants than perivenous parenchymal cells. Chylomicron remnants are cleared from the plasma very rapidly in rats, with a half-life of 1–5 min (Harris and Felts 1970). Thus, *in vivo*, periportal hepatocytes are likely to be exposed to higher concentrations of remnants than perivenous cells, as the microanatomy of the liver is such that blood passes through the periportal parenchyma prior to entering the perivenous zone. It is interesting to note that the acinar heterogeneity of the remnant uptake parallels that of cholesterol *de novo* synthesis (Sultan et al. 1989), cholesterol esterification (unpublished observation), bile formation (Jones et al., 1978; Daoust,

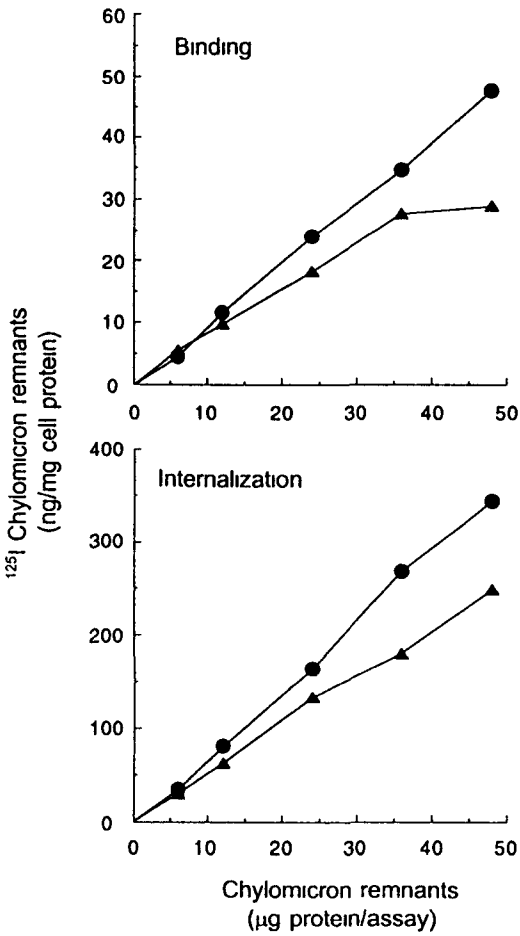

Figure 1. Binding and internalization of ^{125}I chylomicron remnants in periportal and perivenous hepatocytes Periportal (●) and perivenous (▲) cells were incubated at 37°C for 30 min with increasing concentrations of ^{125}I chylomicron remnants, and binding and internalization were measured as described in Materials and Methods The values represent means of three experiments using cells from 6 different rats

1979) and hepatic lipase secretion (Verhoeven and Jansen 1989) The binding of ^{125}I-β-migrating VLDL to rat liver parenchymal cells for 2 h at 4°C was reported to be approximately equal in periportal and perivenous cells (Voorschuur et al 1994) β-VLDL are abnormal lipoproteins that consist of a mixture of cholesteryl ester-rich chylomicron remnants and VLDL remnants, found only in patients with type III hyperlipoproteinaemia and in animals fed diets high in fat and cholesterol

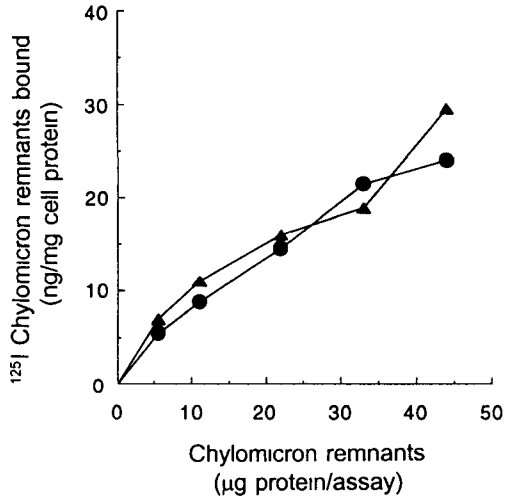

Figure 2. Initial binding of ^{125}I chylomicron remnants in periportal and perivenous hepatocytes Periportal (●) and perivenous (▲) cells were incubated at 4 °C for 2 h with increasing concentrations of ^{125}I chylomicron remnants, and radioactivity associated to cells was measured as described in Materials and Methods The values represent means of two experiments using cells from 4 different rats

(Fainaru et al 1988) There is evidence to suggest that their uptake by the liver is mediated by the remnant receptor (Harkes et al 1989) It is therefore possible that the hepatic processes involved in the cellular recognition and uptake of chylomicron remnants and β VLDL differ in a substantial number of aspects

It has been suggested that the LDL receptor plays the major role in the uptake of chylomicron remnants in rat liver (Choi et al 1991, Jackle et al 1992, Szanto et al 1992, Ishibashi et al 1994) Modulation of the expression of the hepatic receptor protein has been reported to lead to corresponding changes in remnant uptake (Jackle et al 1992, Szanto et al 1992) Nagata et al (1988) found that an anti-LDL receptor antibody inhibited association of the lipoproteins with rat hepatocytes in primary culture A number of studies by van Berkel and colleagues (Harkes and van Berkel 1982, Nagelkerke et al 1986, van Dijk et al 1992), however, have consistently shown that, in normal physiological conditions, the expression of the LDL receptor is very low in rat liver parenchymal cells In our experiments, incubation of periportal or perivenous cells with ^{125}I chylomicron remnants in the presence of an excess of unlabelled human LDL at 37 °C had little effect on either binding or internalization of ^{125}I chylomicron remnants over a range of remnant (Fig 3) and LDL concentrations (Fig 4), and a similar result was obtained when the incubations were carried out at 4 °C (Fig 5) Thus, LDL does not seem to compete

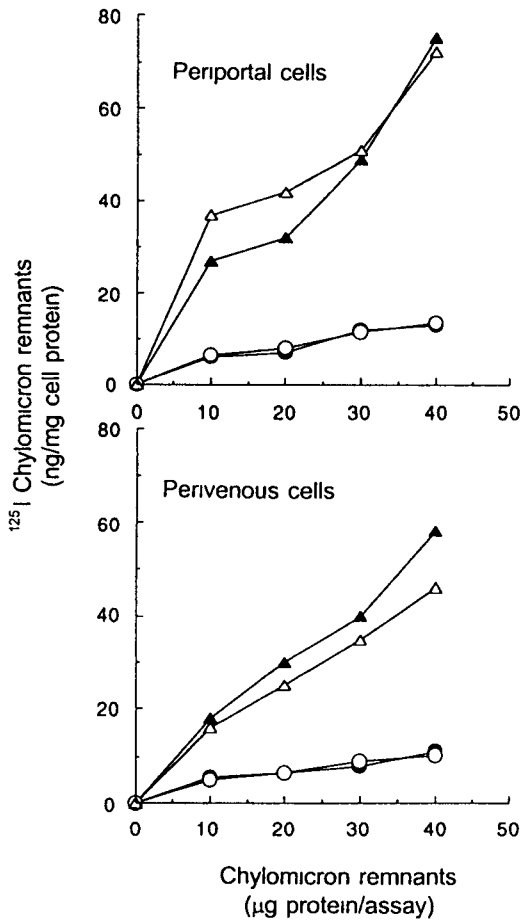

Figure 3. Effect of LDL on the kinetic of ^{125}I chylomicron remnant binding and internalization in periportal and perivenous hepatocytes Periportal and perivenous cells were incubated at 37°C for 30 min with increasing concentrations of ^{125}I chylomicron remnants in the presence (open symbols) or the absence (closed symbols) of LDL (150 μg protein/assay), and binding (●, ○) and internalization (▲, △) were measured as described in Materials and Methods The values represent means of two experiments using cells from 4 different rats

with the remnants for binding or internalization by either of the hepatocyte subpopulations It has been reported previously that LDL is able to compete with chylomicron remnants for binding to rat liver endosomal membranes (Jackle et al. 1992), while the anti-LDL receptor used by Nagata et al. (1988) was found to decrease remnant internalization, but not binding, in rat hepatocyte monolayer

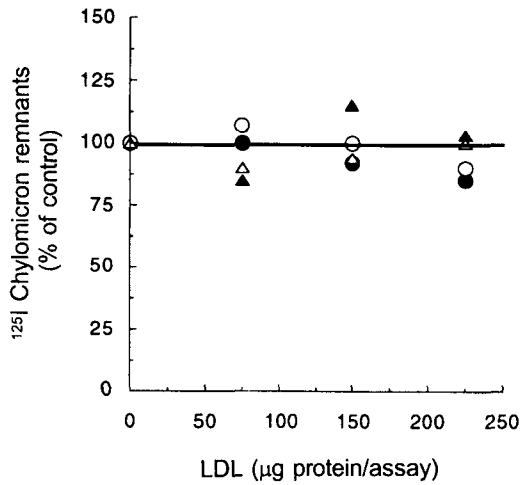

Figure 4. Effect of increasing amounts of LDL on binding and internalization of ^{125}I chylomicron remnants in periportal and perivenous hepatocytes Periportal (●) and perivenous (▲) cells were incubated at 37°C for 30 min with ^{125}I chylomicron remnants (30 μg protein) in the presence of increasing concentrations of LDL, and binding (closed symbols) and internalization (open symbols) were measured as described in Materials and Methods The values represent means of two experiments and are given as a percentage of the values obtained in the absence of LDL

cultures. In agreement with the work reported here, however, the studies of van Dijk et al (1992) indicated that LDL was essentially ineffective as a competitor for chylomicron remnant association with primary rat hepatocytes in suspension when incubations were carried out at both 37°C and 4°C.

The glycoprotein lactoferrin has been shown to contain a sequence resembling the receptor recognition site in apoE (Huettinger et al. 1992), and is known to inhibit specifically the uptake of chylomicron remnants by rat liver cells both *in vivo* and *in vitro* (van Dijk et al. 1992; Huettinger et al. 1992). Evidence from previous work indicates that uptake via the LRP (Huettinger et al. 1992) and LSR (Mann et al. 1995), but not the LDL receptor (van Dijk et al. 1991), is affected by lactoferrin. In our experiments, incubation of periportal or perivenous parenchymal cells with ^{125}I chylomicron remnants in the presence of lactoferrin at 37°C led to inhibition of internalization, but not binding, of the lipoprotein in both cases (Fig. 6). The extent of the decrease in internalization observed was smaller at the higher concentrations of remnants used, and the patterns of inhibition were similar in both subpopulations. At 4°C, the inclusion of lactoferrin in the medium also did not change the initial binding in either cell type (Fig. 5). These results are consistent with those of Huettinger et al. (1992), who found that lactoferrin

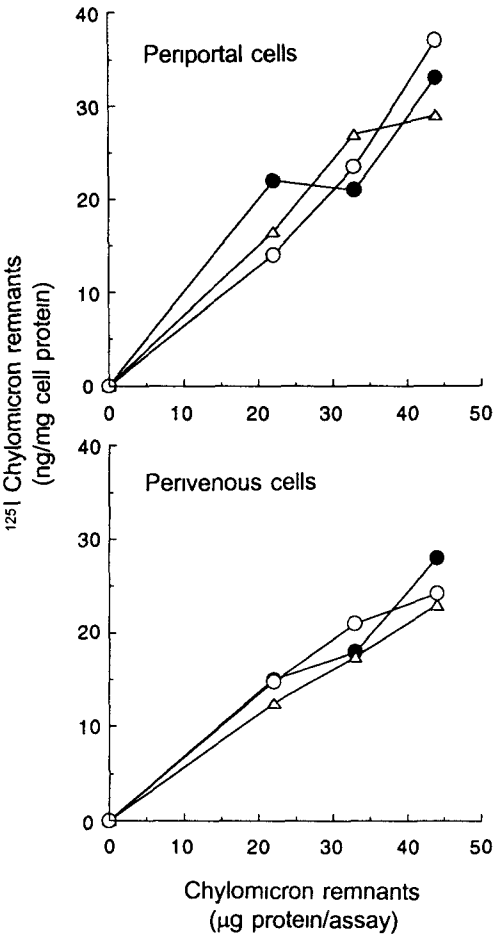

Figure 5. Effect of LDL and lactoferrin on the kinetic of ^{125}I chylomicron remnant initial binding in periportal and perivenous hepatocytes Periportal and perivenous cells were incubated at 4°C for 2h with increasing concentrations of ^{125}I chylomicron remnants in the absence (•) or the presence of LDL (○, 150 μg protein/assay) or lactoferrin (Δ, 4 mg/assay), and initial binding was measured as described in Materials and Methods The values represent means of two experiments using cells from 4 different rats

specifically inhibits endocytosis of chylomicron remnants in rat liver. In addition, van Dijk et al. (1992) have shown that the association of chylomicron remnants with the liver *in vivo* and with isolated hepatocytes *in vitro* decreases in the presence of lactoferrin, although in this case inhibition was found on incubation of the cells at both 37°C and 4°C. We conclude, therefore, that the lactoferrin-sensitive receptor-

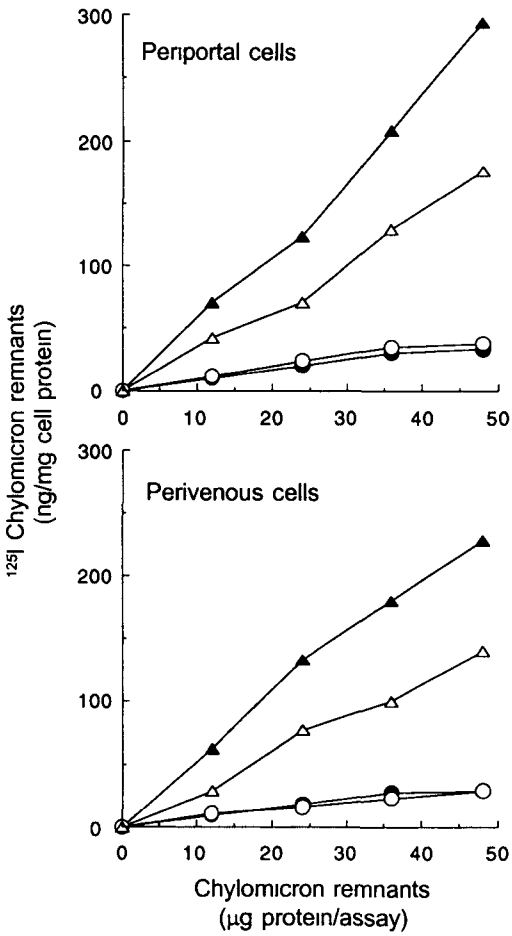

Figure 6. Effect of lactoferrin on the kinetic of ^{125}I chylomicron remnant binding and internalization in periportal and perivenous hepatocytes Periportal and perivenous cells were incubated at 37°C for 30 min with increasing concentrations of ^{125}I chylomicron remnants in the presence (open symbols) or the absence (closed symbols) of lactoferrin (4 mg/assay), and binding (●, ○) and internalization (▲, △) were measured as described in Materials and Methods The values represent means of two experiments using cells from 4 different rats

mediated uptake of chylomicron remnants is similar in periportal and perivenous hepatocytes in normal physiological conditions.

Our results support the idea, proposed recently from studies with a variety of experimental models including mice lacking the LDL receptor, that chylomicron

remnants bind initially to sites on the hepatocyte surface which are distinct from the LDL receptor or the LRP, with the two receptors playing a part in the endocytosis of the particles at a later stage (Herz et al. 1995; de Faria et al. 1996; Krapp et al. 1996). Though in the mouse the LDL receptor was found to have the major role (Herz et al. 1995), our results and those of van Dijk et al. (1992), suggest that the non-LDL receptor mediated uptake, which may involve the LRP or the LSR, is more important in rat liver, and this is consistent with the low expression of hepatic LDL receptors in this species (Harkes and van Berkel 1982; Nagelkerke et al. 1986; van Dijk et al. 1992).

In summary, our findings demonstrate that chylomicron remnants are taken up by similar receptors in periportal and perivenous rat liver parenchymal cells, but the uptake capacity is greater in the periportal than in the perivenous subpopulation Uptake in both cell types was partially blocked by lactoferrin, but not by an excess of LDL, indicating that the LDL receptor has a less important role than the remnant receptor regardless of the hepatic zone of origin of the cells. These results suggest that, like some other aspects of liver cholesterol metabolism, acinar heterogeneity may be a factor in the regulation of the uptake and metabolization of cholesterol from the plasma lipoproteins.

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