The interaction *in vivo* of transferrin and asialotransferrin with liver cells

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Rat transferrin or asialotransferrin doubly radiolabelled with ^{59}Fe and ^{125}I was injected into rats. A determination of extrahepatic and hepatic uptake indicated that asialotransferrin delivers a higher fraction of the injected ^{59}Fe to the liver than does transferrin. In order to determine *in vivo* the intrahepatic recognition sites for transferrin and asialotransferrin, the liver was subfractionated into parenchymal, endothelial and Kupffer cells by a low-temperature cell isolation procedure. High-affinity recognition of transferrin (competed for by an excess of unlabelled transferrin) is exerted by parenchymal cells as well as endothelial and Kupffer cells with a 10-fold higher association (expressed per mg of cell protein) to the latter cell types. In all three cell types iron delivery occurs, as concluded from the increase in cellular $^{59}\text{Fe}/^{125}\text{I}$ ratio at prolonged circulation times of transferrin. It can be calculated that parenchymal cells are responsible for 50–60% of the interaction of transferrin with the liver, 20–30% is associated with endothelial cells and about 20% with Kupffer cells. For asialotransferrin a higher fraction of the injected dose becomes associated with parenchymal cells as well as with endothelial and Kupffer cells. Competition experiments *in vivo* with various sugars indicated that the increased interaction of asialotransferrin with parenchymal cells is specifically inhibited by *N*-acetylgalactosamine whereas mannan specifically inhibits the increased interaction of asialotransferrin with endothelial and Kupffer cells. Recognition of asialotransferrin by galactose receptors from parenchymal cells or mannose receptors from endothelial and Kupffer cells is coupled to active ^{59}Fe delivery to the cells. It is concluded that, as well as parenchymal cells, liver endothelial and Kupffer cells are also quantitatively important intrahepatic sites for transferrin and asialotransferrin metabolism, an interaction exerted by multiple recognition sites on the various cell types.

INTRODUCTION

All cells require iron, and the physiological apparatus for iron delivery utilizes a serum glycoprotein, transferrin (Aisen & Listowsky, 1980). About 10% of iron bound to plasma transferrin is destined for the liver (Polycove & Mortimer, 1961). With many cell types it has been shown that the acquisition of iron requires the expression of cell-surface transferrin receptors to which the iron-loaded protein binds. Subsequently endocytosis of the iron-transferrin complex occurs and iron is removed. In striking contrast with many other endocytosed ligands, transferrin is not degraded (Karin & Mintz, 1981; Octave *et al.*, 1981) and remains bound to its receptor until the receptor rejoins the plasma membrane and releases the apotransferrin (Karin & Mintz, 1981; Octave *et al.*, 1981). This unique mechanism of receptor-mediated endocytosis has been derived from studies with cells in culture, such as K562 cells (Van Renswoude *et al.*, 1982), Hep G2 cells (Ciechanover *et al.*, 1983) and isolated rat hepatocytes (Young *et al.*, 1985) and recently in a perfused liver system (Morgan *et al.*, 1986). A number of groups, however, concluded that receptor-mediated endocytosis does not occur in hepatocytes and that iron uptake occurs by alternative processes such as pinocytosis (Grohlich *et al.*, 1979; Page *et al.*, 1984), diffusion (Page *et al.*, 1984) or removal of iron from transferrin at the

plasma membrane (Cole & Glass, 1983; Veldman *et al.*, 1986). Moreover, the presence of any transferrin receptors on isolated hepatocytes has been questioned, and iron uptake by the liver has been described as a function of endothelial liver cells (Soda & Tavassoli, 1984; Kishimoto & Tavassoli, 1985).

Recently some of us established a system to determine the receptor-dependent interaction of ligands with the various liver cells *in vivo* (Nagelkerke *et al.*, 1983; Van Berkel *et al.*, 1985a). After injection of radiolabelled ligands *in vivo*, a liver perfusion at a low temperature is started (8 °C), a liver lobule is tied off to determine the total liver uptake, and subsequently cell separation and purification procedures are performed at 8 °C in order to determine the association of radioactivity with parenchymal, Kupffer and endothelial liver cells (Nagelkerke *et al.*, 1983; Van Berkel *et al.*, 1985a,b). The low temperature isolation method enables the quantitative recovery of radioactivity cell-associated *in vivo*, in the purified cell fraction. In the present experiments this system is utilized to determine the high-affinity interaction of doubly labelled $^{59}\text{Fe}/^{125}\text{I}$ rat transferrin with the various liver cells *in vivo*. Furthermore, rat asialotransferrin, a glycoprotein with terminal galactose residues, was used as a control ligand because it was expected that asialotransferrin would interact specifically with galactose receptors from hepatocytes (Ashwell &

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earlier (Nagelkerke *et al.* 1983). The total cell isolation procedure is schematically shown in Fig. 1. As found earlier (Harkes & Van Berkel, 1984; Van Berkel *et al.*, 1985a,b; Nagelkerke *et al.*, 1984, 1986) with other protein substrates, no loss of cell-bound or formation of acid-soluble radioactivity occurred from the ^{125}I -labelled (asialo)transferrin during the low temperature cell isolation procedures, leading to a quantitative recovery of the radioactivity associated with total liver in the subsequently isolated cells. This was checked for each individual cell isolation by comparing the calculated (from the relative contribution of the various cell types) and determined total liver association (recovery percentages varied between 92 and 104%). The recovery of ^{59}Fe both from transferrin and asialotransferrin in the isolated cells was significantly lower than the apoprotein (cf Figs. 3 and 5), with percentages ranging from 47 to 62% at 5 min after injection, 37 to 42% at 30 min and 27 to 31% at 120 min after injection. Calculation of the contribution of the different cell types to total liver uptake was performed as described (Nagelkerke *et al.*, 1983). The purity of the cell preparations was furthermore routinely checked light-microscopically after staining for peroxidase and by determination of the distribution of the isoenzymes of pyruvate kinase.

Tissue uptake of (asialo)transferrin

At 30 min after injection of the (asialo)transferrin into rats the tissues were removed, weighed and the amount of radioactivity was determined.

RESULTS

Tissue distribution of transferrin and asialotransferrin

In order to define the tissue specificity of iron uptake from transferrin and asialotransferrin, the percentage of the injected dose associated with various organs and tissues was determined after 30 min of circulation (Figs. 2a and 2b). Only the liver, spleen and bone marrow

showed an increased $^{59}\text{Fe}/^{125}\text{I}$ ratio as compared with the administered transferrin. The highest ratio is found in bone marrow (~ 20). After injection of doubly labelled asialotransferrin a similar uptake pattern is obtained as with transferrin, with however a clearcut increase in uptake by the liver (2-fold).

The time-dependent accumulation in the liver of ^{59}Fe either transported by transferrin or asialotransferrin (Fig. 3), indicates that uptake is already significant 30 min after injection [in these experiments the liver was perfused at a low temperature (8°C), so that the contribution of the blood compartment (approx. 9% of the serum value) (Van Berkel *et al.*, 1985a), is avoided]. From the disparity in the serum decay of ^{125}I -transferrin and ^{59}Fe it is evident that at 15 min after injection iron delivery must already have occurred (Fig. 3). While the ^{125}I -transferrin radioactivity in serum for transferrin and asialotransferrin is comparable, a higher fractional catabolic rate of ^{59}Fe from asialotransferrin is noticed, which can be accounted for by the higher fractional uptake in the liver.

Cellular distribution of (asialo)transferrin in liver

In order to evaluate the cell isolation system as presented in Fig. 1, the cellular uptake of ^{125}I -asialofetuin in the absence and presence of an excess of unlabelled asialofetuin was determined (Fig. 4). At 10 min after injection $77 \pm 3\%$ ($\pm \text{s.e.m.}$, $n = 3$) of the injected dose is present in the liver. Cell-association of ^{125}I -asialofetuin to Kupffer and endothelial cells is about 3-fold higher when expressed per mg of cell protein. Taking into account the amount of protein contributed by each cell type to total liver, the proportion of the total liver uptake of asialofetuin for which each cell type is responsible can be calculated (Nagelkerke *et al.*, 1983). For parenchymal cells this is 82.5%, for endothelial cells 9.3% and for Kupffer cells 8.2%. A high-affinity association (effectively competed for by unlabelled asialofetuin) is only seen with parenchymal cells.

Fig. 2. Recovery sites of rat $^{59}\text{Fe}/^{125}\text{I}$ -transferrin (a) and rat $^{59}\text{Fe}/^{125}\text{I}$ -asialotransferrin (b) at 30 min after injection

Diferric transferrin (0.38 mg of protein) or diferric asialotransferrin (0.38 mg of protein) was injected into anaesthetized rats and at 30 min after injection the rat was killed, rapidly cooled to 4°C and the various tissues were removed, weighed and the radioactivity determined. Values are expressed as $10^4 \times$ percentage of the injected dose/mg wet wt. Hatched blocks represent ^{59}Fe radioactivity and open blocks represent ^{125}I radioactivity.

Fig. 3. Liver association (a) and serum decay (b) of rat $^{59}\text{Fe}/^{125}\text{I}$ -transferrin and rat $^{59}\text{Fe}/^{125}\text{I}$ -asialotransferrin

Labelled transferrin (0.40 mg of protein) or asialotransferrin (0.40 mg of protein) was injected in anaesthetized rats and the serum decay and liver association of ^{125}I -transferrin (○), ^{125}I -asialotransferrin (□), ^{59}Fe -transferrin (●) and ^{59}Fe -asialotransferrin (■) was determined at the indicated times. The decay values are the means of three to five independent experiments with S.E.M. of 1–2%. The livers were perfused for 8 min in order to prevent a contribution of the blood compartment to the liver values.

Fig. 4. Effect of preinjection of asialofetuin on the association of ^{125}I -asialofetuin with parenchymal, endothelial and Kupffer cells

^{125}I -asialofetuin (9 μg) was injected into anaesthetized rats, which were preinjected at -1 min with 5 mg of asialofetuin (B), 25 mg of asialofetuin (C) or the solvent only (A). At 10 min after injection of ^{125}I -asialofetuin a liver perfusion was started, and the total liver association (after an 8 min perfusion at 8 $^{\circ}\text{C}$) and the association with the subsequently isolated parenchymal, endothelial and Kupffer cells were determined. When bars are indicated, these represent S.E.M. ($n = 3$).

The association of ^{125}I -transferrin to the liver is exerted by parenchymal cells as well as endothelial and Kupffer cells (Fig. 5). Surprisingly, for ^{125}I -asialotransferrin as compared to transferrin, a higher fraction of the injected dose becomes cell-associated to all three cell types. At short times after injection, the specific activity (expressed per mg of cell protein) of ^{125}I -transferrin or

^{125}I -asialotransferrin is about 10-fold higher in endothelial or Kupffer cells as compared with parenchymal cells (see also Fig. 6). At prolonged circulation times this factor is even higher (15–20). Recalculation of specific activities to relative contribution to total liver uptake (Table 1) indicates that about half of the total liver-association is exerted by parenchymal cells, about

Fig. 5. Time-dependent association of rat ⁵⁹Fe/¹²⁵I-transferrin and rat ⁵⁹Fe/¹²⁵I-asialotransferrin to parenchymal (a), endothelial (b) and Kupffer (c) cells

Labelled transferrin (0.40 mg of protein) or asialotransferrin (0.40 mg of protein) was injected into anaesthetized rats. At the indicated times a cell separation was started and the cell association of ¹²⁵I-transferrin (○), ¹²⁵I-asialotransferrin (□), ⁵⁹Fe-transferrin (●) and ⁵⁹Fe-asialotransferrin (■) was determined.

Table 1. Relative contribution of the different liver cell types to the total liver uptake of asialofetuin, transferrin and asialotransferrin

The amount of radioactivity/mg of cell protein in the isolated cell fractions was multiplied by the amount of protein that each cell type contributes to total liver protein. The values are calculated for transferrin and asialotransferrin from cell uptake values determined 2 h after injection, while asialofetuin uptake was determined 10 min after injection.

Cell type	Uptake (% of total) of:				
	Asialofetuin	¹²⁵ I-transferrin	⁵⁹ Fe-transferrin	¹²⁵ I-asialotransferrin	⁵⁹ Fe-asialotransferrin
Parenchymal cells	83	44	51	51	53
Endothelial cells	9	32	27	28	35
Kupffer cells	8	24	22	21	12

30% by endothelial cells and 20% by Kupffer cells. These percentages were calculated for asialotransferrin and transferrin based upon the distribution data determined at 120 min after injection. At 5 min after injection these values were 55–67% for parenchymal cells, 17–26% for endothelial cells and 18–22% for Kupffer cells, and at 30 min after injection 53–60% for parenchymal cells, 22–34% for endothelial cells and 15–18% for Kupffer cells.

Effect of asialofetuin and unlabelled transferrin

The role of high-affinity sites for transferrin in the interaction of transferrin with the various liver cells was explored by injection of an excess of unlabelled transferrin 2 min before the labelled ligand. At 5 min after injection of ¹²⁵I-transferrin the cells were isolated and the cell-associated radioactivity was determined (Fig. 6). The association of ¹²⁵I-transferrin with parenchymal, endothelial as well as Kupffer cells is similarly inhibited by an excess of unlabelled transferrin.

Preinjection of 25 mg of asialofetuin inhibited the association of ¹²⁵I-asialotransferrin with the various liver

cells to an interaction value which is identical with that for the sialylated transferrin.

Effect of GalNAc, GlcNAc and mannan

The sugar specificity of the cell-association of ¹²⁵I-asialofetuin and ¹²⁵I-asialotransferrin was investigated by preinjection of sugar competitors. Preinjection of GalNAc blocked the uptake of ¹²⁵I-asialofetuin into parenchymal cells nearly completely (Fig. 7). In contrast, the association with endothelial or Kupffer cells was unaffected by GalNAc, while preinjection of mannan specifically acted on endothelial and Kupffer cells.

The uptake of ¹²⁵I-asialotransferrin by hepatocytes was inhibited more than 52% by preinjection of GalNAc (Fig. 8). It must be kept in mind that the association of transferrin with the cells is about 30% of that of asialotransferrin, so that the maximal sugar-mediated inhibition will be 70%. Similarly, as with ¹²⁵I-asialofetuin, GalNAc does not affect the interaction of ¹²⁵I-asialotransferrin with endothelial or Kupffer cells whereas with these latter cell types mannan is specifically effective.

Fig. 6. Effect of preinjection of transferrin or asialofetuin on the cell association of rat ^{125}I -transferrin or rat ^{125}I -asialotransferrin with parenchymal, endothelial and Kupffer cells

^{125}I -transferrin (TF, 0.40 mg of protein) or ^{125}I -asialotransferrin (ATF, 0.40 mg of protein) was injected into anaesthetized rats. Hatched blocks with TF represent cell-association of ^{125}I -transferrin after preinjection at -2 min of 50 mg of unlabelled transferrin. Hatched blocks with ATF represent cell-association of ^{125}I -asialotransferrin after preinjection of 25 mg of asialofetuin. Open bars represent cell-association with preinjection of the solvent of these substances. The cell separation procedure was started 5 min after injection of the labelled ligand.

Fig. 8. Effect of preinjection of GalNAc, GlcNAc or mannan on the cell association of ^{125}I -asialotransferrin with parenchymal, endothelial and Kupffer cells

^{125}I -asialotransferrin (0.38 mg of protein) was injected into anaesthetized rats which were preinjected at -1 min with 0.5 mmol of GalNAc, 0.5 mmol of GlcNAc, 5 mg of mannan or the solvent of these substances (control). At 5 min after injection of ^{125}I -asialotransferrin the liver cell isolation procedure was started and the values are expressed as percentage of the control.

Fig. 7. Effect of preinjection of GalNAc, GlcNAc or mannan on the cell association of ^{125}I -asialofetuin with parenchymal, endothelial and Kupffer cells

^{125}I -asialofetuin (9 μg) was injected into anaesthetized rats which were preinjected at -1 min with 0.5 mmol of GalNAc, 0.5 mmol of GlcNAc, 5 mg of mannan or the solvent of these substances (control). At 10 min after injection of ^{125}I -asialofetuin the liver cell isolation procedure was started and the values are expressed as percentage of the control.

DISCUSSION

The data indicate that with the present liver cell-isolation procedure, it is possible to determine *in vivo* the specific interaction of transferrin and asialotransferrin with the various liver cell types. The specific interaction of transferrin with the liver endothelial and Kupffer cells per mg of cell protein was found to be at least 10-fold higher than with the parenchymal cells. However, because these cell types only contain 3.3% and 2.5% respectively of the liver protein (Van Berkel *et al.*, 1985a,b) their relative contribution to the total liver uptake does not exceed 35% and 24% respectively. This indicates that liver parenchymal cells are still responsible for more than 44% of the liver interaction with transferrin.

Autoradiographic data of Hershko *et al.* (1973) and Morgan *et al.* (1986) indicated a more prominent role for the parenchymal cells, while Soda & Tavassoli (1984) and Kishimoto & Tavassoli (1985) suggested that only liver endothelial cells should possess transferrin receptors. The maximal number of binding sites for transferrin found with isolated hepatocytes varies between zero and 162000 per cell (Young & Aisen, 1980; Cole & Glass, 1983; Morley & Bezkorovainy, 1983; Thorstensen & Romslo, 1984; Tei *et al.*, 1984), while in a perfused liver system approximately double this number can be calculated if the binding is assumed to be confined to hepatocytes (Morgan *et al.*, 1986). The presence of about half the transferrin receptors in non-parenchymal cells can perfectly explain these discrepancies.

The involvement of a high-affinity site on parenchymal, endothelial and Kupffer cells responsible for the interaction with the transferrin *in vivo* is indicated by the inhibitory effect of an excess of unlabelled transferrin. Furthermore, the high-affinity recognition of transferrin is coupled to iron delivery in all three cell types, with kinetics consistent with separate iron uptake pathways in the various cell types. This contrasts with earlier suggestions for iron delivery to parenchymal cells, for which transcytosis through endothelial cells was suggested to be obligatory (Soda & Tavassoli, 1984; Kishimoto & Tavassoli, 1985). However, it must be remembered that liver parenchymal cells have direct access to the blood compartment, because liver endothelial cells possess fenestrations of about 100 nm (Wisse, 1970), allowing free exchange of sinusoidal blood components to the space of Disse. The quantitative aspects of ^{59}Fe uptake in the isolated cells must however be interpreted cautiously because, in contrast with the apoprotein, part of the ^{59}Fe is apparently lost from the cells during the cell isolation procedure.

The increased fraction of desialylated transferrin which becomes associated with the liver cells is thought to be mediated by a receptor which also recognizes asialofetuin with a high affinity (Ashwell & Morell, 1974). Indeed, we found that an excess of asialofetuin completely abolished the increased interaction of asialotransferrin with the various liver cells. However, the increased interaction of asialotransferrin as compared with transferrin with the endothelial and Kupffer cells was quite unexpected and competition experiments with various sugars were necessary to evaluate the recognition specificity. The increased interaction of asialotransferrin with parenchymal cells was specifically inhibited by GalNAc and not affected by GlcNAc or mannan. A similar inhibitory specificity was noticed with ^{125}I -asialofetuin, indicating that the recognition of asialotransferrin by liver parenchymal cells is mediated by a galactose-specific receptor. However, neither the interaction of asialotransferrin nor asialofetuin with Kupffer and endothelial cells was affected by GalNAc. These data also rule out the possibility that galactose receptors from non-parenchymal cells (Kolb-Bachofen *et al.*, 1982) do interact with asialotransferrin or asialofetuin. However, these cell types do possess mannose-specific receptors (Steer & Clarenburg, 1979; Achord *et al.*, 1978) and competition experiments with mannan indeed indicate the involvement of mannose groups in the interaction of asialotransferrin and asialofetuin with Kupffer and endothelial cells, so also explaining the inhibitory effect of asialofetuin on asialotransferrin binding to these latter cell types (Fig. 6). It is well known that sialic acid removal from carbohydrate chains not only exposes terminal galactose groups for receptor recognition but also subterminal carbohydrate chain components (Hubbard *et al.*, 1979). So the similar subterminal presence of mannose units in asialotransferrin and asialofetuin results in an increased interaction of these ligands with the Kupffer and endothelial liver cells.

The increased fraction of asialotransferrin, as compared with transferrin, which becomes associated with the various liver cells, also leads to an increased ^{59}Fe delivery. Studies with isolated hepatocytes *in vitro* indicated that asialotransferrin and asialofetuin segregate from each other, after cellular uptake, with asialofetuin transport to lysosomes and asialotransferrin recycling to

the cell surface (Regoecezi & Debanne, 1981; Tolleshaug, 1984). Our present data *in vivo* indicate that the increased iron delivery of asialotransferrin to the liver as compared with transferrin does not lead to an equivalent lower serum percentage of ^{125}I -asialotransferrin, so suggesting that also *in vivo* ^{125}I -asialotransferrin can be released from the liver again after cellular delivery of its iron.

In conclusion, it can be stated that the interaction of transferrin and asialotransferrin with the liver involves the concerted action of parenchymal, Kupffer and endothelial liver cells, leading to the necessity of including the multiple cellular interaction sites in future studies on the role of the liver in (asialo)transferrin metabolism.

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