Desialylation of transferrin by liver endothelium is selective for its triantennary chain

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Liver endothelium can remove and transport the glycoprotein transferrin (TF). During this process the molecules are desialylated; however, in contrast with other such glycoproteins, for example caeruloplasmin, only half of transported TF is desialylated. To explore which component of TF is desialylated, we double-labelled fully sialylated TF with [³H]sialic acid residues and a ¹²⁵I-protein moiety. This was then 'chased' through purified liver endothelium in pulse-chase experiments. Endothelium-conditioned TF was fractionated on an RCA₁₂₀ affinity column into sialylated and desialylated components. Each component was then re-fractionated on a concanavalin A affinity column, which separates the glycoprotein according to the branching pattern of its glycan chain. The desialylated fraction was eluted only as a triantennary component, whereas the non-desialylated fraction consisted only of bi- and tetra-antennary chains. The significance of this selective desialylation of triantennary chain of TF in the subsequent metabolism of its iron content and its possible role in the pathogenesis of alcohol-induced hepatic siderosis are discussed.

INTRODUCTION

Transferrin (TF), the iron-binding plasma glycoprotein, has an M_r of about 80000 and contains 6% (w/w) carbohydrate (Huebers & Finch, 1984; Irie & Tavassoli, 1987). In rat TF, the glycan moiety is in the form of a single asparagine-linked oligosaccharide chain (Schreiber *et al.*, 1979). The chain branches at the site of a mannose residue into a biantennary or a triantennary component, with each antenna ending in a galactose residue that may or may not be concealed by a sialic acid residue. TF preparations are therefore heterogeneous, having a mixture of biantennary and triantennary glycan chains, and the proportion of each can vary from one preparation to the other. The degree of sialylation may also be variable, resulting in further heterogeneity (Stibler *et al.*, 1979, 1980; Stibler & Borg, 1981; Regoeczi *et al.*, 1984; Storey *et al.*, 1987).

There is now considerable evidence that capillary endothelia in general, and liver endothelium in particular, are involved in the transport of many circulating proteins into the parenchyma (DeBruyn *et al.*, 1983; Soda & Tavassoli, 1983, 1984a,b; Blomhoff *et al.*, 1984; Kataoka & Tavassoli, 1984, 1985; Moguilevsky *et al.*, 1984; Williams *et al.*, 1984; King & Johnson, 1985; Kishimoto & Tavassoli, 1985, 1987; Pitas *et al.*, 1985; Ghitescu *et al.*, 1986; Simionescu & Simionescu, 1987; Irie *et al.*, 1988). In the same vein, we have demonstrated that Fe-TF complexes can be taken up through a receptor-mediated mechanism by liver endothelium (Tavassoli *et al.*, 1986a,b; Irie *et al.*, 1988) and transported via a vesicular transport system (Tavassoli *et al.*, 1986a,b; Kishimoto & Tavassoli, 1987) across the cell, to be subsequently externalized (Kishimoto & Tavassoli, 1987). This transport path is apparently different from the endosomal pathway, since Fe which dissociates from TF in the low pH of the endosome remains associated with

it in the course of this transport (Irie *et al.*, 1988). During this transendothelial transport, only 55% of TF is desialylated (Irie *et al.*, 1988). The remaining molecules keep their full degree of sialylation. This is in contrast with other such plasma glycoproteins, for example caeruloplasmin (CP), which is also taken up and transported by liver endothelium via a similar vesicular system, but is completely desialylated (Tavassoli *et al.*, 1986b). The reason for this discrepancy is not clear, but partial desialylation of TF may be related to its heterogeneity. In the present work, we have investigated this phenomenon by double-labelling TF and subsequently fractionating it by lectin affinity chromatography. We now provide evidence that desialylation of TF is selective for those molecules with a triantennary glycan chain, and this selectivity could have implications in the subsequent metabolism of the Fe-TF complex in the liver.

EXPERIMENTAL

TF and labelling methods

Purified rat TF was obtained from Cooper (Westchester, PA, U.S.A.). Its purity had been demonstrated by immunoelectrophoresis and was independently confirmed in our laboratory by isoelectrofocusing, which demonstrated two bands (slow and fast) in fully saturated form (Okada *et al.*, 1979). To remove desialylated or partially sialylated TF, the protein was subjected to affinity column chromatography using an RCA₁₂₀-agarose column as described below. This lectin binds terminal galactose residues of desialylated glycoprotein, permitting first the elution of fully sialylated molecules (Pardoe *et al.*, 1968; Nicolson & Blaustein, 1972). Therefore, in all subsequent studies, the fraction that was not retained by the column was used.

Apo-TF was then prepared from TF by sodium

Abbreviations used: TF, transferrin; CP, caeruloplasmin; PBS, phosphate-buffered saline (0.057 M-phosphate/0.163 M-NaCl, pH 7.4); D-PBS, Dulbecco's phosphate-buffered saline (PBS plus Ca²⁺); Con A, concanavalin A; RCA₁₂₀, *Ricinus communis* agglutinin.

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ascorbate as described by Kishimoto & Tavassoli (1985). It was double-labelled with [^3H]sialic acid and ^{125}I -protein. The method for double-labelling of TF has been developed in our laboratory and described elsewhere (Kishimoto & Tavassoli, 1986). Essentially, sialic acid residues of apo-TF were labelled with ^3H by using consecutive oxidation with NaIO_4 and reduction with NaB^3H_4 . The latter step introduces ^3H into the sialic acid residues (Van Lenten & Ashwell, 1971). Apo-TF (5 mg) in 0.5 ml of 0.1 M-sodium acetate/0.15 M- NaCl (1:1, v/v; pH 5.6) was first oxidized by incubation with 0.208 ml of 0.012 M- NaIO_4 for 10 min at 0 °C. Oxidation was stopped by the addition of an excess of ethylene glycol (500 μl), and the solution was dialysed overnight at 4 °C against phosphate-buffered saline (PBS), pH 7.4. Periodate consumption, determined by iodometric titration (Dyer, 1956) indicated that more than 1.5 mol of periodate was oxidized/mol of sialic acid. This is fairly close to 2 mol/mol, which is theoretically ideal. To the oxidized TF solution was added 1 mg of NaB^3H_4 (ICN) dissolved in 0.2 ml of 0.01 M- NaOH . After mixing, the solution was warmed to room temperature and the reduction allowed to continue for 30 min with stirring. To insure the completeness of reduction, 3 mg of non-radioactive KBH_4 were then added and the incubation continued for another 30 min. Excess borohydrate was removed by dialysis against a solution of 0.1 M-sodium acetate/0.15 M- NaCl , pH 5.6, for several hours before exhaustive dialysis against PBS, pH 7.4. Because glucose linked non-enzymically to protein through an aldimine could also be reduced and labelled by this method (Bunn *et al.*, 1979), we subsequently hydrolysed the sialic acid residues (see below) of these preparations by an enzymic method (Van Lenten & Ashwell, 1971). This demonstrated that 91% of the label was on sialic acid residues. Since complete removal of sialic acid residues is not possible, these data suggest that only sialic acid residues in these preparations were labelled.

The protein part of apo-TF was labelled with ^{125}I by using Bolton-Hunter reagent, since the usual peroxidation method could theoretically remove the ^3H label from sialic acid residues. Double-labelled TF was then saturated with Fe as described in detail previously (Kishimoto & Tavassoli, 1985; van Renswoude *et al.*, 1982). Hereafter TF refers to fully saturated diferric holotransferrin unless otherwise specified. The specific radioactivities of ^{125}I and ^3H were 8 and 16 $\mu\text{Ci/nmol}$ respectively.

Chromatography

Native TF, as well as endothelium-conditioned supernatant from pulse-chase studies (see below), were subjected to concanavalin A (Con A)-Sephacrose chromatography. The supernate was dialysed overnight at 4 °C against a buffer, pH 6.9, containing 0.1 M- NaCl , 5 mM-sodium acetate, 1 mM- CaCl_2 , 1 mM- MgCl_2 , 1 mM- MnCl_2 (hereafter referred to as 'Con A-buffer') and concentrated by an Amicon membrane (M_r cut-off > 10000) to 250 μl . It was then subjected to chromatography on a column (10 mm $\times$ 75 mm) of Con A-Sephacrose (Sigma), which was equilibrated with Con A-buffer and eluted, with the same buffer at a flow rate of 5 ml/h at room temperature, as fractions of 1 ml each. The retained fraction was completely eluted with Con A-buffer containing 0.2 M- α -methyl D-glucoside (Sigma).

Radioactivities and the trichloroacetic acid-precipi-

tability were checked in each fraction of both the RCA_{120} and Con A affinity chromatographies.

Cell preparations and incubation

Crude liver-cell suspensions were obtained from Sprague-Dawley rats (150–250 g) by using the collagenase-perfusion method (Seglen, 1976). Cells were suspended in Dulbecco's PBS (D-PBS) to a final concentration of 4×10^6 cells/ml. They were partially fractionated into large- (hepatocytes) and small- (endothelium-rich) cell fractions by using Metrizamide double-layer centrifugation as described (Kataoka & Tavassoli, 1984; Kishimoto & Tavassoli, 1985). To purify endothelial cells further, centrifugal elutriation was used (Kishimoto & Tavassoli, 1985; Zahltén *et al.*, 1978). The endothelium-rich small-cell fraction was suspended in PBS at a concentration of 2×10^7 /ml and loaded in a Beckman JE6-B standard elutriator rotor (4.2 ml); a JS-21 centrifuge was used. Before cell loading, the rotor was eluted with PBS at a flow rate of 11 ml/min. The rotor was then loaded, using a total volume of 4 ml, while maintaining the same flow rate. Elutriation was done at 650 g at 20 °C. The flow rate was increased to 22 ml/min, and 300 ml (corresponding to about 2×10^7 cells) of eluent fluid was collected. Cell identification was made by scanning and transmission electron microscopy, as well as by cytochemistry, as described (Kataoka & Tavassoli, 1984; Kishimoto & Tavassoli, 1985). Endothelial-cell identification was further confirmed by an immunofluorescent reaction for Factor VIII/vW antigen by using the indirect method described by Kishimoto & Tavassoli (1985). The purity of endothelial cells was 92%, with a viability (as determined by Trypan Blue exclusion) of 97%. The viability was determined in several experiments after incubation and did not change.

Endothelial cells (10^6 cells) were first incubated with fully sialylated, double-labelled diferric TF in D-PBS at a concentration of 100 $\mu\text{g}/\text{ml}$, containing 0.5% bovine serum albumin, to a total volume of 250 μl at 4 °C for 30 min. This was done to saturate the surface TF receptors (Kishimoto & Tavassoli, 1985). Cells were then washed thrice with ice-cold D-PBS to remove unbound labelled TF. Surface-bound labelled TF was then chased through the cells by the addition of excess unlabelled TF in microcentrifuge tubes at 37 °C for 60 min with gentle agitation (Irie *et al.*, 1988). At the end of the incubation time each cell suspension was centrifuged and the supernatant containing externalized radiolabelled TF was obtained. Pooled supernatants from several experiments were used for chromatographic studies.

Desialylation and measurement of sialic acid

TF was desialylated by neuraminidase treatment (Van Lenten & Ashwell, 1971) using *Clostridium perfringens* neuraminidase (Sigma). To do so, TF (5 mg/ml) was incubated with 0.5 unit of neuraminidase attached to beaded agarose (Sigma) in a buffer, pH 5.5, containing 0.1 M-sodium acetate with 5 mM- CaCl_2 for 48 h at 37 °C in a mechanical shaker under an atmosphere of toluene. After incubation, neuraminidase was removed by centrifugation. The solution was then subjected to gel filtration to remove free sialic acid using a Sephadex G-25 column (1.2 cm $\times$ 28 cm) equilibrated with PBS, pH 7.4. Elution was done at a flow rate of 6 ml/h with spectrophotometric monitoring of protein concentration, and the eluate was collected in 1.2 ml fractions.

Fig. 1. RCA₁₂₀-agarose and Con A-Sepharose chromatograms of native diferric rat TF double-labelled with either ^{125}I -protein moiety (●) or ^3H -sialic acid residues (○)

(a) RCA₁₂₀-agarose chromatogram of the native protein, obtained as described in the text. The arrow indicates the application of 0.2 M-β-D-galactose. (b) Con A-Sepharose chromatogram of the native protein. The arrow indicates the application of 0.2 M-α-methyl D-glucoside. (c) RCA₁₂₀-agarose chromatogram of the supernatant obtained after the incubation of fully sialylated TF (component I of a) with purified liver endothelium, obtained as described in the text. Note that about 45 % of the applied TF is now totally desialylated (containing no ^3H) and retained by the column, whereas the rest remains sialylated (containing ^3H). The two peaks, as indicated by the black horizontal bars, were collected, concentrated and applied to the Con A column separately. (d) Shows that peak I in (c) (sialylated fraction) gives two peaks corresponding to components I and II (tetra- and bi-antennary) of (b) respectively. (e) Shows that peak II of (c) (desialylated fraction) is eluted in the same position as component II of (b) (triantennary). In this Figure and Fig. 2 values in parentheses indicate the percentages of each eluted peak on a protein basis.

Estimation of free sialic acid after desialylation was done by the thiobarbituric acid method (Kishimoto & Tavassoli, 1986).

RESULTS AND DISCUSSION

On the basis of ^{125}I -labelling, native rat TF gave three peaks on RCA₁₂₀-agarose (Fig. 1a). The first one, with a sialic acid/protein molar ratio of 2.3, was continuous with the second one, which had a ratio of 1.8. The third peak, which was 1.6 % of the applied sample, contained no sialic acid and was eluted only with β-D-galactose. These ratios were corroborated by the relative magnitude of the ^3H curves (representing sialic groups) which in all Figures are shown in correlation with ^{125}I curves (representing the protein moiety). It was evident from both sialic acid/protein ratios and the magnitude of the ^3H curve that the first peak was fully sialylated, the second one only partially sialylated, and the third peak was totally desialylated. Hence, in these studies, TF was first subjected to the RCA₁₂₀-agarose column and the fully sialylated fraction (first peak) was obtained for the subsequent incubation.

Fig. 1(b) shows the elution pattern of native TF on the Con A column. Here, too, three components could be identified. In component I, which was eluted most rapidly and constituted 10 % of the applied sample, the ^3H label exceeded ^{125}I label in relative terms, and the sialic acid/protein ratio measured chemically was 3.5. In component II, which gave a broad elution peak and constituted 60 % of the applied sample, the ^3H label only slightly exceeded the ^{125}I label in the same relative terms. The sialic acid/protein ratio in this component was 2.1. Component III, which gave a sharp elution peak, and was eluted only with α-methyl D-glucoside, constituted 30 % of the applied sample. In this component the ^3H and ^{125}I labels coincided and, by chemical measurement, the sialic acid/protein ratio was 1.8. Reproducibility of these data was confirmed by repeating the experiments three to five times.

Although only one set of results (the most typical) is given, all results were within a similar range. The data are consistent with the interpretation that component I consisted of a tetra-antennary component II of a triantennary, and component III of a biantennary, glycan chain. The affinity for Con A increased as the number of branches decreased, so that the tetra-antennary component was eluted earliest, followed by the triantennary component. By contrast, biantennary molecules bound strongly to the column and required α-methyl D-glucoside for their elution.

When fully sialylated TF, fractionated on the RCA₁₂₀ column (fraction I; Fig. 1a) was desialylated *in vitro* and again fractionated on the RCA₁₂₀ column, about two-thirds of the TF was then retained (Fig. 2a) and required β-D-galactose for its elution, thus confirming that desialylation had indeed occurred. That the desialylation was nearly complete was further evident from the fact that the molecules contained very little ^3H label. When the retained fraction (component III; Fig. 2a) was then subjected to the Con A column, all three components, seen in fully sialylated form of TF, were again observed (Fig. 2b). This suggested that the fractionation on the Con A column was independent of the presence or the number of sialic acid residues and that the pattern of the

Fig. 2. RCA₁₂₀-agarose chromatogram of native diferric TF before and after desialylation

(a) TF was double-labelled with ¹²⁵I-protein moiety (●) and [³H]sialic acid residues (○) and subsequently desialylated *in vitro*. Conditions of chromatography are those described in the text. The arrow again indicates the application of β-D-galactose. (b) Con A chromatogram of the retained (desialylated) fraction III, as indicated by the black horizontal bar in (a). The Con A chromatogram is identical with that of fully sialylated protein (Fig. 1b).

terminal branching of the glycan chain of the molecule was the determinant of their affinity for Con A.

When fully sialylated TF (component I, Fig. 1a) was incubated with purified endothelial cells in a pulse-chase experiment and the supernatant was then subjected to RCA₁₂₀ columns, two peaks were observed (Fig. 1c). The first one, constituting 55% of the ¹²⁵I label, was eluted early and contained ³H label, indicating the absence of desialylation of this fraction by endothelium. The second peak, constituting 45% of the ¹²⁵I radioactivity, was devoid of ³H label. This component was retained by the column and could only be competitively eluted by β-D-galactose, suggesting that complete desialylation had occurred after incubation with endothelium.

When the sialylated fraction of this supernatant (component I, Fig. 1c) was separated, concentrated and subjected to Con A-Sepharose chromatography, two peaks were obtained with ¹²⁵I radioactivity (Fig. 1d), corresponding to components I and III of Fig. 1b (tetra-antennary and biantennary components of fully sialylated TF). When the desialylated fraction of the supernatant (component II, Fig. 1c) was similarly subjected to Con A-Sepharose chromatography, a single radioactive peak was obtained (Fig. 1e), co-migrating

with the component II (Fig. 1b, triantennary component) of fully sialylated TF.

There is increasing evidence to indicate that liver endothelium has receptors for a number of plasma proteins and can transport them across the cell via receptor-mediated transcytosis (Blomhoff *et al.*, 1984; Moguilevsky *et al.*, 1984; Kataoka & Tavassoli, 1985; Pitas *et al.*, 1985; Tavassoli *et al.*, 1986a,b; Kishimoto & Tavassoli, 1987; Irie *et al.*, 1988). In the case of the two glycoproteins that we have thus far studied (TF and CP), endothelium can desialylate them (Irie *et al.*, 1988; Irie & Tavassoli, 1986). This is evident by separately labelling the protein moiety with ¹²⁵I and the sialic acid residues with ³H. Pulse-chase experiments then showed that although the two labels are taken up concordantly, their behaviour then becomes discordant: ¹²⁵I exits from the cell, whereas ³H is retained (Tavassoli *et al.*, 1986a,b; Irie *et al.*, 1988). Moreover, the radioactive proteins that exit from the cell now have the electrophoretic mobility of asialoglycoproteins (Tavassoli *et al.*, 1986a,b; Irie *et al.*, 1988). In addition, endothelially-processed protein now binds to the RCA₁₂₀ column, indicating that the sialic acid residues have been removed to expose the penultimate galactose residues. Therefore the elution of protein from the column requires competitive elution with β-D-galactose. Desialylated molecules can subsequently be removed via asialoglycoprotein receptors of hepatocytes (Tavassoli *et al.*, 1986a,b).

Of interest is that, whereas CP is totally desialylated (Irie & Tavassoli, 1986), only about half of the TF is desialylated (Irie *et al.*, 1988). To examine the reason for this discrepancy, in the present work, fully sialylated TF was fractionated on an RCA₁₂₀ column and then subjected to Con A chromatography. Con A recognizes mannose residues located at the branching point of the glycan chain. Although its highest affinity is for mannosidic and hybrid chains, it also has a fairly high affinity for biantennary chains and retains these molecules, their removal requiring competitive elution. Its affinity for tri- and tetra-antennary chains is weaker (Ogata *et al.*, 1975; Krusius *et al.*, 1976; Hodges *et al.*, 1979; Yamamoto *et al.*, 1981). Thus, by Con A chromatography, fully sialylated rat TF gave three peaks that, by chemical-measurement of sialic acid, show sialic acid/protein ratios of 3.5, 2.1 and 1.8, which can be interpreted as being tetra-, tri and bi-antennary molecules. Although these ratios do not correspond exactly to the theoretical ratios, they are close enough to be consistent with this interpretation. Moreover, this interpretation is also consistent with the magnitude of the ³H curve relative to the ¹²⁵I curve. That the tetra-antennary and biantennary components on the Con A column gave sharp peaks, while the triantennary component gave a broad one, may be explained by the fact that the latter, in contrast with the former, is non-symmetrical in its branching. The presence of a tetra-antennary chain in rat TF apparently has not been reported before.

However, the affinity of Con A for variably branched glycan chains does not depend on its sialic acid content, but only on the pattern of branching. This is evident in Con A chromatographic pattern of TF desialylated *in vitro*, a pattern that is similar with fully sialylated preparations.

Noteworthy in the present study is the clear-cut evidence that the desialylation of TF by liver endothelium is selective for its triantennary component. Tetra-

antennary and biantennary components are not desialylated by endothelium. It is unknown at what level this selectivity is actually exerted. It is possible that triantennary molecules are actually selectively removed by the cells (owing to, e.g., avidity of receptors) or selectivity is exerted at the cellular level where desialylation actually takes place. That CP, in contrast with TF, is totally desialylated, may be related to the fact that the molecule has four glycan chains, many of them being triantennary (Jamieson, 1965; Yamashita *et al.*, 1981). It is therefore probable that the difference in the configuration of the glycan substrate, rather than the endothelial sialidase, determines which molecules are to be desialylated; triantennary molecules having asymmetrical glycan branching are recognized by the enzyme, whereas molecules having symmetrical glycan branching are not susceptible to desialylation.

The significance of this finding may be in the subsequent metabolism of TF by hepatocytes. There is now considerable evidence that alcoholism can enhance desialylation of serum TF (Stibler *et al.*, 1979, 1980; Stibler & Borg, 1981; Regoeczi *et al.*, 1984; Storey *et al.*, 1987). Increased desialylated TF has been utilized as objective evidence of alcoholic liver disease and, moreover, it has been implied in the pathogenesis of alcohol-induced hepatic siderosis. It is possible that alcohol can also alter the ratio of triantennary to other components of TF. The desialylated fraction of TF can be subsequently recognized and taken up by hepatocyte through its membrane asialoglycoprotein receptors. These receptors possess selectivity for the triantennary glycan chain of asialoglycoproteins (Ashwell & Morell, 1974; Baenziger, 1985; Regoeczi & Koj, 1985). On the other hand, TF molecules containing a biantennary (and tetra-antennary) glycan chain are not desialylated by the endothelium and could be recognized by hepatocyte through receptors specific for the protein core of the molecule (Aisen & Listowski, 1980; Young & Aisen, 1980). The intracellular pathways followed by these two types of receptors and ligands may be different, and the relative proportion of these two pools may have implications in iron metabolism by the liver. Iron delivered to hepatocytes via asialotransferrin (containing triantennary glycan) is likely to be sequestered in a less mobilized form by hepatocytes, since asialoglycoproteins are degraded within the cell (Klausner *et al.*, 1983). This may ultimately lead to hepatic siderosis. On the other hand, sialylated TF (biantennary or tetra-antennary glycan) is internalized via TF receptors and is recycled, and iron could thereby be re-utilized rather than sequestered (Klausner *et al.*, 1983). It is therefore possible that alcohol can change the proportion of triantennary molecules and induce their desialylation, which can then shift more iron via asialoglycoprotein receptors of hepatocytes to the less-mobilizable pool, ultimately leading to hepatic siderosis (Irie & Tavassoli, 1987). This scheme, still hypothetical, is nonetheless quite capable of being experimentally tested.

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