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The effects of an antibody to the rat transferrin receptor and of rat serum albumin on the uptake of diferric transferrin by rat hepatocytes

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The role of high-affinity specific transferrin receptors and low-affinity, non-saturable processes in the uptake of transferrin and iron by hepatocytes was investigated using fetal and adult rat hepatocytes in primary monolayer culture, rat transferrin, rat serum albumin and a rabbit anti-rat transferrin receptor antibody. The intracellular uptake of transferrin and iron occurred by saturable and non-saturable mechanisms. Treatment of the cells with the antibody almost completely eliminated the saturable uptake of iron but had little effect on the non-saturable process. Addition of albumin to the incubation medium reduced the endocytosis of transferrin by the cells but had no significant effect on the intracellular accumulation of iron. The maximum effect of rat serum albumin was observed at concentrations of 3 mg/ml and above. At a low incubation concentration of transferrin (0.5 μM), the presence of both rat albumin and the antibody decreased the rate of iron uptake by the cells to about 15% of the value found in their absence, but to only 40% when the diferric transferrin concentration was 5 μM. These results confirm that the uptake of transferrin-bound iron by both fetal and adult rat hepatocytes in culture occurs by a specific, receptor-mediated process and a low-affinity, non-saturable process. The low-affinity process increases in relative importance as the iron-transferrin concentration is raised.

Introduction

In previous studies from this laboratory it was shown that cultured hepatocytes from adult and 19-day gestational fetal rats can accumulate iron from diferric transferrin and incorporate it into ferritin [1,2]. Similar results have been reported for isolated adult hepatocytes by other investigators [3]. Hepatocytes [1,4–8], like other cells which have specialised roles in iron metabolism, such as developing erythroid cells [9] and placental cells [10] possess high-affinity transferrin binding sites which are believed to mediate the uptake of transferrin-bound iron. However, direct evidence for this is lacking in hepatocytes.

In addition, hepatocytes also mediate the uptake of diferric transferrin by a non-saturable, low-affinity process which results in a net accumulation of iron over transferrin [1,5,6,8,11,12]. However, the functional importance of this iron uptake mechanism is uncertain. Some workers have reported that it can be eliminated when plasma proteins other than transferrin, such as albumin [13,14], immunoglobulins [13] and caeruloplasmin [14] are added to the cell incubation medium. In contrast, others have found that transferrin and

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iron uptake by the low-affinity process persists even in the presence of bovine serum albumin [1,5,8].

The aim of this study was to determine the roles of the high- and low-affinity transferrin uptake processes in the uptake of iron by hepatocytes. The specificity of the two processes was investigated by measuring transferrin and iron uptake by cultured adult and fetal rat hepatocytes in the absence and in the presence of rat serum albumin and/or an antibody to the rat transferrin receptor. The results confirm the presence and quantitate the importance of specific, high-affinity transferrin receptors in iron uptake by the cells. They also confirm the functional importance of a low-affinity or non-saturable uptake process and demonstrate that it increases in importance as the diferric transferrin concentration of the incubation medium is raised.

Materials and Methods

Protein purification and labelling. Rat transferrin was isolated from pooled rat plasma using ion-exchange and gel-filtration chromatography [15]. Albumin was purified from the albumin-rich fractions obtained during the preparation of rat transferrin by the method of Michael [16]. The purity of these proteins was assessed by SDS-polyacrylamide gel electrophoresis [17]. Both proteins migrated as single bands. The transferrin was 95% saturated with iron labelled with ^{59}Fe using the iron chelator nitrilotriacetate [18], to give a specific activity of approximately 0.3 μCi ^{59}Fe/nmol transferrin. The iodine monochloride method [19] was used to label the transferrin with ^{125}I to give a final specific activity of 0.8 μCi ^{125}I/nmol protein and approximately 0.5 mole iodine per mole transferrin. The radioisotopes ($^{59}FeCl_3$ and Na ^{125}I) were obtained from Amersham International, Amersham, U.K.

Hepatocyte isolation and culture. Fetal hepatocytes were isolated from the fetuses of 19-day gestation pregnant Wistar rats and maintained in culture as described previously [1]. Adult hepatocytes were isolated from male rats (6–8 weeks old) by a modification of the method of Seglen [20] and maintained in culture as described previously [12] except that the incubation medium was replaced at 4 h with Minimal Essential Medium (Flow Laboratories, N. Ryde, NSW, Australia) containing the synthetic medium Ultroser G (2% v/v; IBF, Pointet Girard, Villeneuve La Garenne, France) instead of fetal calf serum. Adult hepatocytes were studied after 24 h in culture while the fetal cells were used on the third day of culture when they had formed a non-proliferating, nearly confluent monolayer which contained less than 1% cells which were not hepatocytes, as determined by staining for the presence of rat serum albumin [21]. Cell viability in all cultures was always greater than 90% as measured by Trypan blue exclusion.

Transferrin receptor antibody. Transferrin receptors were isolated from the placentas of 19-day gestation pregnant rats and used to produce an antiserum in rabbits as previously described [22]. The IgG-containing fraction was then extracted using ammonium sulphate precipitation and ion-exchange chromatography [15]. Specificity of the antiserum was assessed by Ouchterlony double immunodiffusion and electrophoretic immunofixation. There was no cross-reactivity with rat transferrin.

Experimental procedures. The majority of the experiments were performed with fetal hepatocytes, but adult hepatocytes were also used in certain experiments to determine whether the observed effects were found with both types of cells. The uptake of transferrin and iron was measured by incubation the cells in Minimal Essential Medium containing ^{125}I-^{59}Fe-diferric transferrin at 37°C. Where appropriate, rat albumin (1–5 mg/ml) was added to the incubation medium. Following incubation with the radiolabelled transferrin, the medium was aspirated and the cell monolayer washed five times with ice-cold balanced salt solution [23]. To separate membrane-bound and intracellular ^{125}I-transferrin and ^{59}Fe the cells were then incubated for 30 min at 4°C with pronase (Boehringer Mannheim, Mannheim, F.R.G.) at a concentration of 1 mg/ml [1,9]. This procedure also released the cells from the culture dish. The cell suspensions were transferred by Pasteur pipettes to microfuge tubes, centrifuged for 15 s at 7000 × *g* (Beckman Microfuge B) and the cellular and supernatant fractions were separated and counted for radioactivity in a three-channel gamma scintillation counter (Packard Tri-

carb Model 5360). An aliquot of the cell fraction was also taken for DNA estimation [24]. To determine the effect of the rabbit anti-rat transferrin receptor antibody on the uptake of transferrin and iron by the hepatocytes, the cells were preincubated with the IgG fraction of the antiserum for 30 min at 37°C before incubation with ^{125}I-^{59}Fe-transferrin at 37°C and then treated with pronase as described above.

The uptake of iron and transferrin by the hepatocytes was calculated from the measured radioactivity in the cell fractions and the specific activity of ^{125}I and ^{59}Fe used. To correct for differences in cell numbers between culture dishes,iron and transferrin uptake is expressed as pmole of iron or transferrin per μg DNA.

Results

The effect of an antibody to the rat transferrin receptor on the uptake of transferrin and iron by the fetal hepatocytes was investigated by incubation the cells in the absence and presence of increasing concentrations of the antibody prepara-

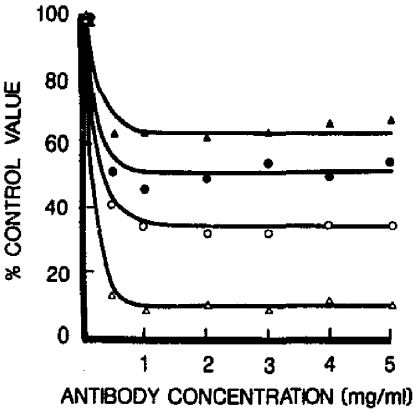

Fig. 1. The effect of anti-rat transferrin receptor immunoglobulin concentration on the membrane and intracellular uptake of transferrin and iron by the fetal rat hepatocytes. The cells were incubated with an anti-transferrin receptor antibody in the concentration range 0.5–5.0 mg/ml for 30 min at 37°C and reincubated with ^{125}I-^{59}Fe-transferrin (1.25 μM) for 2 h. The cells were then incubated with 1 mg/ml pronase for 30 min at 4°C and centrifuged to separate surface-bound and intracellular radioactivity. The means from duplicate estimations of uptake in the presence of the antibody are expressed as a percentage of uptake measured in its absence. Membrane-bound transferrin (●) and iron (▲), intracellular transferrin (○) and iron (△). Similar results were obtained in two experiments of this type.

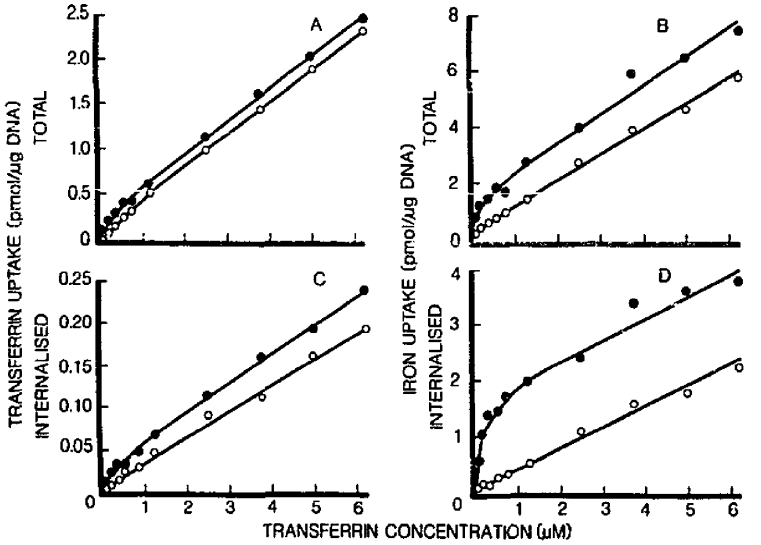

Fig. 2. The effect of an antibody to the rat transferrin receptor on total transferrin (A), total iron (B), internalised transferrin (C) and internalised iron (D) uptake by fetal hepatocytes. The cells were preincubated in the presence or absence of the immunoglobulin (1 mg/ml) for 30 min at 37°C and reincubated with increasing concentrations of ^{125}I-^{59}Fe-transferrin (0.125–6.25 μM) for 4 h at 37°C). The cells were then treated with pronase as described in Fig. 1 to measure total and internalised radioactivity. Results are means of duplicate measurements of uptake by the control cells (●) and by the cells preincubated with the antibody (○). Similar results were obtained in two experiments of this type.

tion (0.5–5 mg/ml) prior to incubation with ^{125}I-^{59}Fe-transferrin (1.25 μM). Treatment of the cells with the antibody reduced transferrin and iron uptake to minimum values when the antibody concentration was at least 1 mg/ml (Fig. 1). The amounts of transferrin and iron bound to the cell surface decreased by approximately 50% and 40%, respectively, so that the iron to transferrin molar ratio in this cellular fraction increased to slightly above 2. The binding of the antibody to the cells reduced the endocytosis of transferrin to 40% and the intracellular accumulation of iron to 10% of the value found in its absence. Hence, the intracellular iron to transferrin molar ratio was reduced from the control value of approximately 20 to 6.

When the fetal cells were incubated with increasing concentrations of radiolabelled transferrin (0.125–6.26 μM) total and intracellular transferrin and iron uptake values increased rapidly in a curvilinear manner until the transferrin concentration reached approximately 1 μM and thereafter more slowly in a linear manner (Fig. 2). These results indicate that uptake was occurring by two processes, a high-affinity, saturable one at transferrin concentrations below 1 μM and a non-saturable one at higher concentrations.

Preincubation of the cells with the antibody reduced transferrin and iron uptake at all transferrin concentrations (Fig. 2A and B). When the transferrin concentration was less than 0.5 μM, transferrin uptake was reduced by the antibody to less than 65% of the control value, while iron uptake decreased to less than 40% of the control value. However, when the transferrin concentration was raised above this level, the amount of transferrin and iron taken up by the treated cells increased as the extracellular concentration was raised to at least 6.25 μM. The slopes of the graphs for transferrin and iron uptake by the antibody-treated cells in the transferrin concentration range of 1.0–6.25 μM were similar to those of the control cells. That is, no further inhibition of transferrin and iron uptake occurred in this higher concentration range. As the uptake of transferrin and iron at higher transferrin concentrations occurs mainly by a non-saturable process, these results demonstrated that the binding of the antibody to the fetal cells did not affect the total uptake of diferric transferrin by this mechanism.

Similarly, incubation of the fetal hepatocytes with the antibody reduced the intracellular uptake of transferrin and iron (Fig. 2C and D). At transferrin concentrations below 0.5 μM, the intracellular uptake of iron was almost abolished while transferrin endocytosis was reduced to less than 40% of the value obtained with the cells not treated with the antibody. When the transferrin concentration was increased above that required to saturate the high-affinity process, there was no

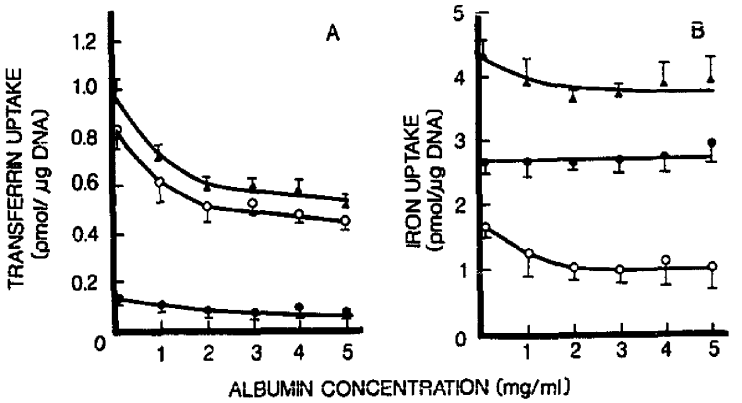

Fig. 3. The effect of rat serum albumin on transferrin (A) and iron (B) uptake by fetal hepatocytes. The cells were incubated for 4 h at 37 °C with ^{125}I-^{59}Fe-transferrin (5.0 μM) in the absence and presence of increasing concentrations of albumin (1–5 mg/ml). Total uptake (▲), internalised uptake (●), surface-bound uptake (○). These results are the means ± S.E. from three estimations. Similar results were obtained in three other experiments of this type.

further inhibition of transferrin and iron internalisation.

The uptake of transferrin by the fetal hepatocytes was reduced by the addition of rat serum albumin to the cell incubation medium (Fig. 3A). The degree of inhibition of transferrin uptake was dependent on the concentration of albumin used, with uptake decreasing as the albumin concentration was increased to approximately 3 mg/ml. Above this concentration, there was no further change in transferrin uptake. Total transferrin uptake was reduced to approx. 60% of the uptake measured in the absence of albumin. This reduction in total uptake resulted mainly from a decrease in the values for surface-bound transferrin, but there was also a reduction in the intracellular uptake to approx. 60% of the control.

In contrast to the results for transferrin, irrespective of the concentration of albumin used there was no significant reduction ($P > 0.05$) in intracellular uptake of iron (Fig. 3B). However, the amount of iron bound to the cell surface was decreased to a minimum value of approx. 60% of the control value when the albumin concentration was at least 3 mg/ml. In the absence of albumin, the iron to transferrin molar ratio in the cell membrane fraction was approx. 2 and it was not altered by the addition of albumin. This indicates that the decrease in iron uptake in this cellular fraction resulted from a reduction in the binding of diferric transferrin to the cell surface.

The studies illustrated in Fig. 3 were performed at a transferrin concentration of 5 μM at which level the uptake of transferrin and iron by both saturable and non-saturable processes occurs. To differentiate between effects of albumin on these two types of processes, fetal and adult cells were incubated with low (0.5 μM) or high (5.0 μM) concentrations of ^{125}I-^{59}Fe-transferrin in the presence or absence of albumin (5 mg/ml). With both types of cells there was little change in the uptake of iron but transferrin uptake decreased in the presence of albumin. However, this decrease was independent of the concentration of transferrin used (Table I). The amount of iron taken up by the fetal cells at both transferrin concentrations was greater than by adult hepatocytes, as observed previously [1,4,5,12]. However, when the uptakes of transferrin and iron in the presence of albumin were expressed as a percentage of uptake in the absence of albumin, as shown in Table I, the effect of albumin on transferrin and iron uptake by the hepatocytes at the two stages of maturity were similar at both transferrin concentrations.

If the hepatocytes were incubated with the antibody to the transferrin receptor (1 mg/ml) to-

TABLE I

THE EFFECT OF RAT SERUM ALBUMIN (RSA) AND AN ANTI-RAT TRANSFERRIN RECEPTOR ANTIBODY (TRA) ON TRANSFERRIN AND IRON UPTAKE BY FETAL AND ADULT HEPATOCYTES

The hepatocytes were incubated in the presence or absence of 1 mg/ml anti-rat transferrin receptor antibody at 37 °C for 30 min and then reincubated with either 0.5 or 5.0 μM ^{125}I-^{59}Fe-transferrin in the presence of 5 mg/ml rat serum albumin for 4 h at 37 °C. The cells were treated with pronase (1 mg/ml) as described in Materials and Methods. Results shown are mean ± S.E. with the number of measurements shown in parentheses.

Experimental conditions	Transferrin concn. (μM)	Iron uptake (% control)		Transferrin uptake (% control)	
		internalised	total	internalised	total
Fetal hepatocytes					
RSA	0.5	95 ± 19 (5)	92 ± 10 (7)	77 ± 9 (5)	73 ± 8 (7)
	5.0	99 ± 12 (9)	93 ± 11 (10)	70 ± 9 (9)	75 ± 10 (9)
RSA + TRA	0.5	15 ± 2 (4)	37 ± 3 (4)	42 ± 5 (4)	65 ± 9 (3)
	5.0	37 ± 13 (3)	63 ± 7 (3)	68 ± 12 (3)	89 ± 4 (3)
Adult hepatocytes					
RSA	0.5	112 ± 9 (5)	110 ± 6 (4)	77 ± 11 (5)	83 ± 19 (4)
	5.0	97 ± 12 (5)	95 ± 12 (5)	62 ± 14 (3)	80 ± 13 (3)
RSA + TRA	0.5	15 ± 2 (6)	30 ± 7 (6)	52 ± 4 (6)	67 ± 6 (6)
	5.0	37 ± 7 (4)	63 ± 8 (4)	71 ± 11 (4)	83 ± 6 (3)

gether with rat serum albumin (5 mg/ml), there was a reduction in uptake and endocytosis of both transferrin and iron (Table I). The degree of inhibition for the adult and the fetal cells was similar, with uptake being reduced to a greater extent at a transferrin concentration of 0.5 μM than 5.0 μM. At the lower transferrin concentration (0.5 μM), the endocytosis of iron and transferrin were reduced to approx. 15% and 45% of the control values, respectively, while at the higher transferrin concentration (5.0 μM) internalised iron and transferrin were reduced to only 40% and 70%, respectively (Table I).

Discussion

This study confirms the presence of high-affinity transferrin receptors on both the fetal [1] and adult hepatocytes [4–8] and demonstrates that they are involved in the uptake of transferrin-bound iron. The uptake of transferrin and iron occurred by a saturable and a non-saturable process (Fig. 2). An antibody to the rat transferrin receptor inhibited the uptake of transferrin and iron only by the saturable process (Fig. 2). The binding of diferric transferrin to the hepatocyte surface was reduced by the antibody (Fig. 1) showing that the antibody was able to inhibit diferric transferrin binding to the transferrin receptor. The decrease in binding of diferric transferrin to the surface transferrin receptors was accompanied by a decrease in intracellular accumulation of transferrin and iron by the cells (Figs. 1 and 2). In contrast, the endocytosis of transferrin and the uptake of iron by the second, non-saturable process was not affected by the antibody to the transferrin receptor (Fig. 2). These results provide direct evidence that the high-affinity receptors mediated transferrin endocytosis and iron uptake by the saturable process and that the non-saturatable process is independent of these receptors.

The specificity of the anti-receptor antibody for the rat transferrin receptor has been confirmed using rat immature erythroid cells. The uptake of transferrin and iron by these cells occurs only by a saturable process [9]. The same antibody preparation as that used in the present work inactivated the transferrin receptors of rat immature erythroid cells, inhibiting transferrin endocytosis and completely blocking iron uptake [25].

In other studies using isolated adult hepatocytes, the proteolytic enzyme, Pronase, was used to inactivate the hepatic transferrin receptors [4,13]. Results obtained using this technique were used as evidence that the uptake of iron by hepatocytes occurs partly by a transferrin receptor-mediated process. The present results confirm this conclusion by the use of a more specific method of inactivating transferrin receptor than that provided by proteolytic enzymes.

Addition of the transferrin receptor antiserum to the cells caused a greater degree of inhibition of iron uptake than transferrin uptake by the hepatocytes (Figs. 1 and 2, Table I). The reason for this may be because a greater proportion of transferrin than iron uptake by the cells involves non-specific and fluid-phase uptake of diferric transferrin [1]. However, the nature of the measurements of iron and transferrin uptake are also different. The uptake of iron is linear throughout the period of incubation due to the continued recycling of labelled transferrin molecules through the cells with release of iron from its carrier molecule and accumulation by the cells [1]. In contrast, the uptake of ^{125}I-transferrin rapidly reaches a maximum or plateau level when a steady state has been reached between the uptake and release of labelled transferrin by the cell [1]. Hence, any treatment of the cells which causes a reduced recycling rate of transferrin through the cells will lead to a reduced rate of iron uptake, but may cause little or no reduction in the steady state level of uptake of labelled transferrin after incubation times of 2 or 4 h, as used in the present work. The fact that some reduction in transferrin uptake was observed indicates that a marked reduction in the rate of receptor cycling and/or inhibition of the interaction of transferrin with its receptor had occurred, but the results do not allow for differentiation between these possibilities.

The experiments with rat serum albumin were performed to assess whether non-specific binding of transferrin plays a role in the intracellular uptake of iron by hepatocytes. At a low transferrin concentration when iron uptake occurs mainly via the transferrin receptor-dependent process, the uptake of iron was not altered by the addition of rat serum albumin to the incubation medium confirming that this mechanism of iron uptake is

specific for transferrin (Table I). At a transferrin concentration of 5 μM, when the non-saturable uptake process predominates, rat albumin partially reduced transferrin uptake, presumably by reducing non-specific adsorption of diferric transferrin to the cell surface and non-specific endocytosis of transferrin. However, the intracellular accumulation of iron was not significantly affected (Fig. 3, Table I). This finding is consistent with other studies in which fetal rat [1] and adult mouse hepatocytes in culture [5] and the isolated perfused rat liver [8] were found to take up transferrin and iron by the low-affinity process even in the presence of bovine serum albumin. This appears to contrast with the results of a previous study using fresh rat hepatocyte suspensions [13]. However, this study reported only total transferrin and iron uptake. Hence, it is not possible to assess whether albumin affected intracellular uptake of iron.

Fetal hepatocytes take up iron at a greater rate than adult hepatocytes [1,4,5,12]. However, the effects of the anti-rat transferrin receptor antibody and rat albumin on the uptake of transferrin and iron by the hepatocytes at both stages of development were similar (Table I). This suggests that in the presence of high concentrations of albumin, as found in vivo, both fetal and adult hepatocytes would take up iron by the high- and low-affinity mechanisms and the relative importance of these two processes in the delivery of iron to the cells would be similar to that found in vitro. The amount of iron taken up by the saturable, high-affinity transferrin receptor-mediated process is limited and is dependent on the number of transferrin receptors and the cycling time of the receptor through the cell. However, the uptake of iron by the low-affinity, non-saturable process is a function of the transferrin-iron concentration. Thus, the influx of iron into the hepatocyte by this process reflects the plasma iron concentration and increases when the plasma iron becomes elevated, as may occur in several haematological disorders and diseases associated with iron overload.

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