Increase in Surface Expression of Transferrin Receptors on Cultured Hepatocytes of Adult Rats in Response to Iron Deficiency*

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The effect of changes in cellular iron metabolism on the surface expression of transferrin receptors (TfR) was examined in primary cultures of hepatocytes from adult rats. Untreated control hepatocytes exhibited a single class of high affinity receptors for transferrin (KD = 40 nM), with approximately 17,000-18,000 receptors per cell. Following 24 h of treatment with the iron chelator, desferrioxamine, or with succinylacetone, an inhibitor of heme synthesis, the number of TfR at the cell surface was increased severalfold, with no significant change in receptor affinity (KD) for transferrin. When combined, the enhancing effects of the two agents were additive. Inhibition of protein synthesis by cycloheximide abolished the increase in TfR expression mediated by either agent. Hemin decreased surface TfR expression and counteracted the enhancing effects of desferrioxamine or succinylacetone on TfR expression. These results indicate that, under the culture conditions employed, 1) iron deficiency induces an increase in surface TfR and 2) modulation of the receptor population is mainly dependent on de novo synthesis of TfR.

It is well established that cellular iron uptake is largely dependent on receptor-mediated endocytosis of transferrin (1), and that replicating cells up-regulate the transferrin receptor (TfR) in response to iron deficiency (2). Hepatocytes, being second to erythropoietic cells in the extent of their synthesis of heme (3) play a central role in systemic iron metabolism. However, the importance of TfR in the uptake of iron by the hepatocytes is unclear. Since there is a high degree of unspecific association of ligands with hepatocytes, it has been difficult to measure the surface expression of high affinity TfR and to assess whether their number is responsive to an increase in cellular iron requirement, as is known to occur in replicating cells (4-10). TfR on human hepatocytes have been recently shown to disappear during hepatic iron overload (11). Such a finding is in agreement with the finding of an inverse relationship between TfR expression and exogenous iron supply in various established, nonhepatic, cell lines (10, 12).

It was the purpose of the present study to inquire whether the number of TfR of hepatocytes in primary culture is up-regulated during cellular iron deprivation as would be expected if transferrin played a significant role in the entry of iron into the liver cell.

EXPERIMENTAL PROCEDURES

Materials

[55S]Sodium iodide (IMS 30) was purchased from Amersham Corp. Collagenase type IV, dexamethasone, insulin, streptomycin, penicillin, hemin, aminolevulinic acid and bovine serum albumin (A 6003) were obtained from Sigma. Tin protoporphyrin was from Porphyrin Products (Logan, UT). Culture medium M199 with Earle's salts was obtained from Boehringer (Mannheim, Federal Republic of Germany); desferrioxamine from Ciba Geigy (Basel, Switzerland) and 4,6-dioxoheptanoic acid (succinylacetone) from Behring Diagnostics. Enzymobead was supplied by Bio-Rad.

Methods

Rat transferrin was purified from serum by the method of Youn and Aisen (13). Diferferic transferrin, obtained by anion exchange and gel filtration chromatography, was rechromatographed until the absorption ratio 470/410 nm was 1.2 or higher. The degree of purity was further verified by polyacrylamide gel electrophoresis. Diferferic transferrin was iodinated with [125I]Sodium iodide using Enzymobead. The physiological integrity of radioiodinated transferrin was confirmed by determination of the iron-donating capacity to rat reticulo-lytes of an aliquot of 5Fe-transferrin iodinated under identical conditions. Unbound radioiodine was reduced to 1-2% by chromatography on Sephadex G25 and dialysis against 0.9% NaCl for several days. The specific activity of 125I-diferferic transferrin obtained by this method was 10,000-25,000 dpm/pmol.

Aminolevulinic acid synthase was measured in the 10,000 X g supernatant of cell homogenates by the method of Sinclair and Granick (14) and heme oxygenase was determined in the 10,000 X g supernatant according to Sardana et al. (15) using, in each instance, 1.2 X 10⁷ cells per assay.

Cell Culture—Hepatocytes were prepared from male Sprague-Dawley rats, fed ad libitum with Altromin standard diet (Lage, FRG), by collagenase perfusion under sterile conditions (16). A 3.0 ml cell suspension, containing 2.2 X 10⁶ hepatocytes, was plated in 60-mm Falcon dishes. The cells were cultured under air/CO₂ (19/1) in medium 199 with Earle's salts containing bovine serum albumin (2 g/liter), NaHCO₃ (20 mM), HEPES (10 mM), streptomycin sulfate (117 mg/liter), penicillin (90 mg/liter), insulin (1 nM) and dexamethasone (10 nM) as described (17). 5% fetal calf serum was present only during the plating phase up to 4 h. After 24 h the medium was replaced with or without additions for a further 24 h (treatment period).

Treatments—The additions to the cultures were desferrioxamine at 0.1, 0.2, 0.5, and 1.0 mM, succinylacetone at 0.5 and 1.0 mM, salicylaldehyde isonicotinohydrazine (a gift from Dr. Premysl Ponka at the Hospital General Juif-Sur Mortimer David in Montreal, Quebec) saturated with equimolar iron at 10-50 uM, aminolevulinic acid at 5.0-500 uM, and heme at 10-50 uM. Heme was quickly dissolved in a small volume of 100 mM NaOH and then in a 50-fold excess of medium. Heme and Fe-salicylaldehyde isonicotinohydrazine at concentrations above 50 uM were toxic to the hepatocytes.
Cycloheximide was added to a final concentration of 5-10 μM, concentrations which had been previously established to have no effects on cell morphology for up to 24 h of treatment (18).

**Transferrin Surface Binding Studies**—At the conclusion of the 24-h treatment period, cells were washed twice with medium, incubated at 37 °C for 60 min to eliminate pre-formed proteins, and then washed twice in medium at 4 °C. The kinetics of transferrin binding to hepatocytes was measured at 0-4 °C to minimize the effects of receptor recycling and metabolism. Hepatocytes were incubated under constant shaking at 60 cycles/min at 0-4 °C in medium containing 3-48 ng/ml (0.7-90 x 10^{-8} M) 125I-diferric transferrin for 120 min, at which time binding had reached equilibrium. When the effects of the various agents on binding parameters were assessed, they were added at concentrations previously determined to be effective. Nonspecific binding was obtained from a parallel set of incubations containing a 50-fold excess of nonradioactive transferrin. Incubations were terminated by washing the cell monolayers with six changes of ice-cold phosphate-buffered saline, pH 7.4. Binding of 125I-transferrin was measured by scraping and transferring cells into test tubes which were counted in a Kontron MR 480 (Munich, FRG) gamma counter.

The experiment was verified by recovery of greater than 95% of the radioactivity when added singly to cultured cells, induced a severalfold increase in total 125I-transferrin binding (Fig. 1); when added in combination, the enhancing effects of these agents on surface 125I-transferrin binding to hepatocytes were additive at lower concentrations of transferrin (Table I). Treatment of hepatocytes with heme for 24 h reduced cellular binding of 125I-transferrin to less than control levels (Fig. 1). Simultaneous exposure of cells to heme together with either desferrioxamine or succinylacetone decreased or abolished the increased binding seen with desferrioxamine or succinylacetone alone (Table I, Fig. 1). The less apparent inhibitory effect of heme on the TIR expression stimulated by desferrioxamine and succinylacetone seen at low concentrations of transferrin probably reflects the nonspecific binding of ligand under noncompetitive conditions. These data reveal that desferrioxamine and succinylacetone act singly and additively to augment surface TIR expression and that heme counters the enhancing effects of these agents.

Fe-salicylaldehyde isonicotinoyl hydrazone, aminolevulinic acid, or tin protoporphyrin exerted no apparent effects on transferrin binding levels (data not shown).

To assure that these agents were exerting an intracellular effect on heme metabolism, the activities of aminolevulinic acid synthase, the first and rate-limiting enzyme of heme biosynthesis, and of heme oxygenase, the rate-limiting enzyme of heme degradation, were measured in treated and untreated cells (Fig. 2). The rate of aminolevulinic acid formation in control hepatocytes was 0.15 nmol/mg of protein/h. Exposure to 1 mM succinylacetone for 24 h increased aminolevulinic acid synthase activity by approximately 7-fold (Fig. 2A). As expected, 30 μM heme exerted product inhibition, reducing the aminolevulinic acid synthase activity to unmeasurable levels; 30 μM tin protoporphyrin, expected to result in heme accumulation, had a similar but less pronounced effect. Fe-salicylaldehyde isonicotinoyl hydrazone, which bypasses the transferrin delivery pathway to make iron

**RESULTS**

Various agents which disturb hepatic iron and/or heme metabolism were used to assess their effects on the concentration of surface TIR in cultured hepatocytes. These agents included an iron chelator (desferrioxamine), an iron chelate (Fe-salicylaldehyde isonicotinoyl hydrazone), a precursor or intermediate in heme synthesis (aminolevulinic acid), inhibitors of heme synthesis (succinylacetone) and heme catabolism (tin protoporphyrin), and heme. Desferrioxamine or succinylacetone, when added singly to cultured cells, induced a severalfold increase in total 125I-transferrin binding (Fig. 1); when added in combination, the enhancing effects of these agents on surface 125I-transferrin binding to hepatocytes were additive at lower concentrations of transferrin (Table I). Treatment of hepatocytes with heme for 24 h reduced cellular binding of 125I-transferrin to less than control levels (Fig. 1). Simultaneous exposure of cells to heme together with either desferrioxamine or succinylacetone decreased or abolished the increased binding seen with desferrioxamine or succinylacetone alone (Table I, Fig. 1). The less apparent inhibitory effect of heme on the TIR expression stimulated by desferrioxamine and succinylacetone seen at low concentrations of transferrin probably reflects the nonspecific binding of ligand under noncompetitive conditions. These data reveal that desferrioxamine and succinylacetone act singly and additively to augment surface TIR expression and that heme counteracts the enhancing effects of these agents.

Fe-salicylaldehyde isonicotinoyl hydrazone, aminolevulinic acid, or tin protoporphyrin exerted no apparent effects on transferrin binding levels (data not shown).

![Graph](image.png)

**FIG. 1.** Percent of transferrin binding to surface transferrin receptors of hepatocytes in culture treated with heme, desferrioxamine (DF), or succinylacetone (SA). Cells were incubated with 2 μg/ml diferric 125I-transferrin at 4 °C as described under "Experimental Procedures." Binding of transferrin to untreated cells (100%) was compared with those treated with 10 or 50 μM heme, 0.1-1.0 mM desferrioxamine, or 0.1 mM succinylacetone. A shows data obtained with either heme, desferrioxamine, or succinylacetone treatments. B shows the combined treatments of heme and desferrioxamine at the two concentrations employed for each agent (A). C shows the combined treatments of heme and succinylacetone at the two concentrations employed for each agent. Data were the average of duplicate determinations of two to five experiments performed with different hepatocyte cultures. The data were statistically analyzed by Student's t test for experiments performed more than twice. The significance level of the data is *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; and +, p > 0.05.

**TABLE I**

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**Additive effect of 24-h treatments with 0.2 mM desferrioxamine (DF) and 1 mM succinylacetone (SA) on surface expression of TIR on rat hepatocytes**

Reversal of desferrioxamine effect by 30 μM heme. The data are an average of values from duplicate dishes of a representative experiment.
available for heme synthesis in reticulocytes,² was found to increase aminolevulinic acid synthase activity. Although our data showed a 67% increase in aminolevulinic acid synthase activity induced by Fe-salicylaldehyde isonicotinoyl hydrazine, statistical analysis of the averages of duplicate samples of four experiments suggested that it was not statistically significant (p > 0.05). Iron chelation treatment with desferrioxamine for 24 h had no effect on aminolevulinic acid synthase activity (Fig. 2A). The rate of bilirubin formation in control hepatocytes was 1.18 nmol/mg of protein/h. Only the treatments with heme and succinylacetone significantly increased the heme oxygenase activity (Fig. 2B).

Kinetic analyses of TfR parameters, obtained with two independent methods of analysis of ¹²⁵I-dextran binding to hepatocytes at 0-4 °C is shown in Fig. 3. Untreated hepatocytes were found to possess 17,000-18,000 surface TfR per cell by both saturation curve analysis (Fig. 3A) and by Scatchard plot (Fig. 3B). The apparent binding affinity (Kd) of the receptor for diferric transferrin was 3.5 × 10⁻⁶ M. Pretreatment of cells for 24 h with 1 mM succinylacetone or 1 mM desferrioxamine significantly increased the concentration of TfR at the cell surface but had minimal effects on the apparent binding affinity (Kd) of the TfR. Thus, after 24 h incubation with succinylacetone the number of surface receptors increased nearly 2-fold to approximately 29,000 receptors per cell. Iron depletion by desferrioxamine resulted in a nearly 5-fold increase in surface TfR to approximately 85,000 receptors per cell (Fig. 3). These results confirmed the enhancement effect of desferrioxamine and succinylacetone on surface TfR concentration (Fig. 1) and exclude the possibility that increased ligand binding resulted from an increased receptor affinity for the ligand.

The addition of cycloheximide to cells incubated with either desferrioxamine or succinylacetone virtually abolished the augmentation of surface TfR induced by these agents (Fig. 4). These data indicate that the enhanced expression of surface TfR associated with desferrioxamine or succinylacetone treatment is dependent upon de novo synthesis of receptor protein.

² P. Ponka, personal communication.

DISCUSSION

The present work demonstrates that adult hepatocytes in primary culture possess a high affinity TfR which is upregulated by increased cellular iron demand. The number of surface TfR detected in our studies with primary cultures of hepatocytes from adult rats was relatively low, amounting to approximately 17,000-18,000 receptors per cell. Several previous studies with freshly isolated hepatocytes from adult rats had indicated that hepatocytes exhibit a relatively low number of surface TfR with an affinity ranging from 10⁻¹⁰ to 10⁻¹¹ M⁻¹. Young and Aisen (13) reported a total TfR number of approximately 34,700 (Kd = 1.62 × 10⁻⁶ M⁻¹) for diferric transferrin. Tavassoli and co-workers have challenged these findings by ascribing transferrin binding to endothelial cells instead of hepatocytes or Kupffer cells (19) and have suggested a mechanism of transendothelial transport of iron-transferrin.
Mitochondrial and microsomal hemeproteins. Following 24-h incubations with 1 mM desferrioxamine (○), desferrioxamine plus 5 μM cycloheximide (●), 1 mM succinylacetone (△), succinylacetone plus 5 μM cycloheximide (▲) or no additions (■), the cells were incubated for 90 min at 4°C with 125I-transferrin at 3, 6, 12, and 24 μg/ml and processed as described under "Experimental Procedures." The data are corrected for nonspecific binding, and the values represent the mean of two experiments.

(20). However, Vogel et al. (21) demonstrated on liver cells, separated by discontinuous iso-osmotic Percoll gradient, an average surface TIR number of 21,000 for hepatocytes (K 1.0 × 10^11 mol−1) and of 5,000 for nonparenchymal cells. The total TIR number of either cell type was twice that of the surface receptor number. These data, obtained with different 111I-rat transferrin, were confirmed by immunocytochemical observations. Our finding of 17,000–18,000 surface TIR per cell on cultured hepatocytes thus confirms the results obtained by previous investigators (13, 21).

Our results also indicate that the surface expression of the TIR is directly related to hepatic iron requirements. Not only was the increase in TIR number observed in the presence of desferrioxamine dose-dependent, but the extent of the increase in general exceed that reported by others for cell lines (10, 12, 22–24). No monoclonal antibody is yet available for the rat TIR, as it is for the human TIR (24), to verify that hepatocyte receptor synthesis rather than externalization had occurred. However, the fact that cycloheximide abolished the increase indicates that this modulation of the receptor population is due to de novo synthesis of TIR molecules during iron deprivation. This finding is in agreement with the observation of a desferrioxamine-induced increase in translatable mRNA for the TIR in K562 cells (23). Clearly, it was the TIR number, not the affinity of the TIR for transferrin which changed during iron chelation treatment (Fig. 3). Such a sensitivity of hepatocytes to the intracellular iron need is not unexpected, as these mitotically quiescent cells (25) are, nevertheless, extraordinarily active in the metabolism of xenobiotics and hormones. For optimal cellular performance the availability of iron would be essential for the synthesis of mitochondrial and microsomal hemeproteins.

The increase in TIR number in hepatocytes noted in response to augmented iron need is indicative of a hitherto undocumented significance for transferrin-mediated iron delivery in liver cells. Sibille et al. (26) have suggested that the iron delivery process mediated by circulating ferritin is a significant alternative pathway of iron uptake by the liver cells under physiological conditions. In circumstances where iron availability becomes limiting, as in iron deficiency, transferrin may serve to augment provision of iron to the liver cell in response to cellular iron demand. The increased TIR expression observed in iron-deficient hepatocytes demonstrates the plasticity of the transferrin-mediated iron delivery pathway and its role in ensuring an adequate supply of iron for the metabolic needs of hepatocytes.

Succinylacetone significantly increased TIR expression in HeLa cells, leading to the conclusion that the availability of heme is the critical determinant regulating TIR expression (12). In our experiments, succinylacetone was only effective at 1 mM and no dose dependence could be established (data not shown). It is possible that succinylacetone, because of its β-diketone structure, may act as an iron chelator. In support of this possibility is the finding that the desferrioxamine-mediated increase in TIR is augmented by succinylacetone when 0.2 mM desferrioxamine (Table I), but not when 1.0 mM desferrioxamine (data not shown), is added. The different results obtained at the two chelator concentrations may, however, reflect the fact that higher concentrations of chelator may induce maximal inhibition of heme synthesis, thereby obviating the added effect of succinylacetone inhibition. Further work is needed to determine whether succinylacetone is modulating TIR expression in hepatocytes through its action on a chelatable iron pool or via its inhibition of heme synthesis.

The finding that heme counteracts the desferrioxamine- and succinylacetone-mediated stimulation of TIR expression confirms results obtained by other investigators (12, 24). The mechanism by which heme exerts its effect on TIR expression in hepatocytes is unclear since iron release from heme during the prolonged course of treatment cannot be excluded. Further studies may confirm our tentative conclusion that both heme and intracellular iron may regulate TIR expression in hepatocytes.

REFERENCES