

Identification and Characterization of the Hepatic Stellate Cell Transferrin Receptor

Kim R. Bridle,*[†] Darrell H.G. Crawford,*[‡]
and Grant A. Ramm*[†]

From the Hepatic Fibrosis Group,* The Queensland Institute of Medical Research and the Department of Medicine,[†] The University of Queensland; and the Departments of Gastroenterology and Hepatology,[‡] Princess Alexandra Hospital, Brisbane, Queensland, Australia

Activated hepatic stellate cells have been implicated in the fibrogenic process associated with iron overload, both in animal models and in human hemochromatosis. Previous studies have evaluated the role of ferritin/ferritin receptor interactions in the activation of stellate cells and subsequent fibrogenesis; however, the role of transferrin in hepatic stellate cell biology is unknown. This study was designed to identify and characterize the stellate cell transferrin receptor and to evaluate the influence of transferrin on stellate cell activation. Identification and characterization of the stellate cell transferrin receptor was determined by competitive displacement assays. The effect of transferrin on stellate cell activation was assessed using western blot analysis for α -smooth muscle actin expression, [³H]Thymidine incorporation, and real-time RT-PCR for procollagen α 1(I) mRNA expression. A specific receptor for rat transferrin was observed on activated but not quiescent stellate cells. Transferrin significantly increased the expression of α -smooth muscle actin, but caused a decrease in proliferation. Transferrin induced a significant increase in procollagen α 1(I) mRNA expression. In conclusion, this study has demonstrated for the first time a specific, high affinity receptor for rat transferrin on activated hepatic stellate cells, which *via* interaction with transferrin regulates stellate cell activation. This suggests that transferrin may be an important factor in the activation of hepatic stellate cells in conditions of iron overload. (Am J Pathol 2003, 162:1661–1667)

Hemochromatosis is a common, genetic iron overload disorder characterized by iron accumulation within the parenchyma of various organs, in particular the liver. Without treatment hemochromatosis can progress to hepatic injury, fibrosis and cirrhosis. The activated hepatic stellate cell (HSC) has been shown to be responsible for fibrosis, which occurs in a variety of chronic liver diseases.^{1–3} Previous studies by our group and others have

demonstrated an association between increasing hepatic iron stores and the presence of the activated HSC phenotype in both C282Y hemochromatosis⁴ and in animal models of iron overload.^{5,6} However, the initiating events responsible for HSC activation in iron overload are not fully understood.

The pathways of iron metabolism in both hepatocytes and Kupffer cells have been extensively studied^{7–9}; however, there is a relative paucity of information on the pathways associated with HSC iron metabolism. HSC have been shown to possess a specific, high-affinity receptor for the iron-storage protein tissue ferritin¹⁰; however, the existence of a receptor for the iron-transport protein transferrin and its role in HSC activation have not been previously examined. As the liver is the predominant site of iron accumulation in hemochromatosis and the site of often severe iron-induced fibrotic injury, an increased understanding of the pathways of iron metabolism in HSC is of great importance in elucidating the mechanisms of iron-induced liver injury.

Transferrin is a single-chain glycoprotein of molecular weight of approximately 80 kd.^{11,12} The transferrin receptor (TfR) is a dimer of subunits of approximately 90 kd each, which are linked by a single disulphide bond. Uptake of iron by hepatic cells through the transferrin/transferrin receptor pathway is considered quantitatively the most important¹³; however, this pathway has not previously been examined in HSC.

The aims of this study were to identify the existence of a HSC transferrin receptor, characterize the binding kinetics of this receptor, and examine the influence of transferrin on parameters of HSC activation. Iron metabolism in HSC is poorly understood and these studies are the first to investigate the kinetics of transferrin uptake and the role of transferrin in the HSC activation process. This study was designed to provide important new knowledge on the pathways associated with HSC iron metabolism and to elucidate the potential mechanisms associated with iron-induced HSC activation, which occurs in diseases of iron overload, such as hemochromatosis.

Supported by an Institute Block Grant from the National Health and Medical Research Council of Australia to The Queensland Institute of Medical Research (to G.A.R. and D.H.G.C.).

Accepted for publication February 5, 2003.

Address reprint requests to Grant A. Ramm, Ph.D., Hepatic Fibrosis Group, The Queensland Institute of Medical Research, Post Office, Royal Brisbane Hospital, Herston, QLD, 4029, Australia. E-mail: grantr@qimr.edu.au.

Materials and Methods

HSC Isolation

Rat HSC were isolated from male Sprague-Dawley rats by sequential pronase and collagenase digestion and separated on an arabinogalactan gradient as previously described.¹⁰ HSC were maintained in M199 (supplemented with 12 mmol/L sodium bicarbonate, 12 mmol/L glucose, 4 U/L insulin, 100,000 U/L penicillin, 0.1 g/L streptomycin, 1 μ mol/L corticosterone and 10 mmol/L HEPES) + 20% horse and calf serum. For uptake experiments this culture media was replaced with supplemented M199 + 1% calf serum. HSC purity at isolation was between 90 and 95% and viability was >95%. By 5 days in culture the purity and viability had reached 97% and 99%, respectively. HSC viability and purity were assessed as previously described by Ramm et al.¹⁴ These studies were approved by the ethics committees of The Queensland Institute of Medical Research and The University of Queensland, Brisbane and complied with guidelines outlined in "Guide for the Care and Use of Laboratory Animals."

Preparation of Proteins

Rat apo-transferrin was purchased from the Sigma Chemical Company (St. Louis, MO) and holo-transferrin was prepared as described previously.¹⁵ To assess iron loading of transferrin the absorbance was measured, assuming that diferric transferrin has an A_{465}/A_{280} ratio of 0.046 and an A_{465}/A_{412} ratio of >1.4.

For all competitive displacement experiments, rat transferrin (RTf) was iodinated using ¹²⁵I-labeled Bolton and Hunter reagent (NEN Research Products, Boston, MA) as described previously.¹⁶

Optimization of ¹²⁵I-RTf Concentration

To determine the optimal concentration of ¹²⁵I-RTf for use in competitive binding experiments, HSC were incubated with increasing concentrations of ¹²⁵I-RTf for 2 hours at 37°C. HSC were plated at 1×10^6 cells/ml and cultured for 5 days in media containing 20% serum. Before the optimization experiment the culture media was replaced by media containing 1% calf serum. HSC were exposed to increasing concentrations of ¹²⁵I-RTf (0.1 to 5 μ g/ml) \pm 50-fold excess of unlabelled RTf for 2 hours at 37°C. Cells were washed three times in ice-cold PBS and lysed by addition of 0.2 N NaOH. Cell-bound ¹²⁵I-RTf was determined using a gamma counter (Packard, Canberra, Australia).

Competitive Binding Assays

After isolation, HSC were plated onto 12-well tissue culture plates at 1×10^6 cells/ml. At days 1, 2, 3, 5, and 7 post-isolation, uptake of ¹²⁵I-RTf was determined as described above with 0.5 μ g/ml ¹²⁵I-RTf \pm 50-fold excess of unlabelled RTf.

To determine whether quiescent HSC express a TfR, freshly isolated HSC were also plated onto Teflon-coated inserts (Millipore Corporation, Bedford, MA) and cultured for 6 hours and 24 hours before incubation with ¹²⁵I-RTf \pm 50-fold excess of unlabelled RTf. A minimum 6-hour culture period post-enzyme digestion was used to ensure recovery of receptors post-digestion. Previously a 2-hour period postdigestion showed recovery of hepatocyte transferrin receptors.¹⁷ Culture on Teflon wells ensures that HSC are maintained in a quiescent phenotype. To confirm that HSC were quiescent after culture on Teflon inserts, cell lysates were collected for α -SMA determination by Western blotting. Viability of HSC cultured on Teflon inserts was also examined by trypan blue exclusion and by incubating cells on plastic following seven days of culture on Teflon. By trypan blue exclusion, viability remained over 90%. Following 7 days of culture on Teflon wells and subsequent culture on plastic, cells remained viable and spontaneously activated as assessed by Western blotting for α -smooth muscle actin (α -SMA; see below)(results not shown).

Transferrin Receptor Specificity

After 5 days in culture, HSC were incubated with 0.5 μ g/ml ¹²⁵I-RTf in M199 + 1% calf serum. Cells were incubated with ¹²⁵I-RTf \pm 50-fold excess of unlabelled RTf, mouse transferrin, bovine serum albumin, rat liver ferritin and rat heart ferritin for 2 hours at 37°C. Cells were washed three times in ice-cold PBS and lysed by addition of 0.2 N NaOH. Cell-bound ¹²⁵I-RTf was determined using a gamma counter.

Time Course Study

After 5 days in culture HSC were washed well in PBS (three times) and incubated with 0.5 μ g/ml ¹²⁵I-RTf \pm 50-fold excess of unlabelled RTf in M199 + 1% calf serum for up to 180 minutes. Bound ¹²⁵I-RTf was determined as described above.

Scatchard Analysis

After 5 days in culture, HSC were incubated with 0.5 μ g/ml ¹²⁵I-RTf \pm increasing concentrations of unlabelled RTf (0.005–150 μ g/ml) in M199 + 1% calf serum. Cells were incubated for 2 hours at 37°C. After this incubation cells were washed, lysed, and bound ¹²⁵I-RTf determined. Scatchard analysis was performed using the LIGAND program as described by Munson and Rodbard.¹⁸

Effect of Transferrin on HSC Activation

Preparation of Whole Cell Lysates and Western Blotting

Freshly isolated HSC were treated with holo-Tf (0.05–0.3 mg/ml) at days 0, 3, and 5 and cell extracts were harvested at day 7. HSC whole-cell lysates were prepared as described previously.¹⁹ Protein concentra-

tion was determined using Peterson's modification²⁰ of the micro-Lowry method (Protein Assay Kit; Sigma Diagnostics, St. Louis, MO). Determination of α -SMA protein expression was achieved by SDS-PAGE and Western blotting. Proteins were transferred to PVDF membrane (Bio-Rad, Regents Park, Australia) before immunodetection with the α -SMA antibody (α -SMA, Sigma Chemical Company; 1:2000). Membranes were then incubated in the secondary antibody (anti-mouse immunoglobulin, horseradish peroxidase [HRP] conjugated, Silenus, AMRAD, Richmond, Australia; 1:1000) before chemiluminescent detection with the ECL detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

[³H]Thymidine Incorporation Assay

Freshly isolated HSC were treated with holo-Tf (0.05–0.5 mg/ml) at days 0, 3, and 5 and cell extracts were harvested at day 7. After 6 days in culture 2 μ Ci/ml [methyl-³H]thymidine (5 Ci/mmol; 1 mCi/ml; Amersham) was added to each well as previously described²¹ and cell lysates were collected at day 7. Lysates were counted in 4 ml of scintillation fluid (Emulsifier-Safe; Packard) in a LKB 1219 rackbeta liquid scintillation counter.

Determination of Procollagen $\alpha_1(I)$ Gene Expression

For mRNA analysis, cells were either treated with RTf at day 0 and RNA collected at day 1, 2, and 3 post-isolation, or grown in culture until day 5, then treated with RTf and RNA collected at 6 and 48 hours post-treatment. HSC were treated with 0.2 mg/ml holo-RTf. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), as per manufacturer's instructions. Concentration and purity of isolated RNA was assessed by spectrophotometry at A260/A280. Following RNA isolation, DNase 1 digestion and first-strand cDNA synthesis were performed using 2 μ g of total RNA and oligo dT15 (Invitrogen). The resulting cDNA was then diluted 2.5-fold.

Real-time RT-PCR was performed to quantitate the expression of procollagen $\alpha_1(I)$ mRNA and the house-keeping gene, β -actin, for each sample. A Rotorgene thermal cycler (Corbett Research, Brisbane, Australia) was used for real-time RT-PCR experiments. Reactions were performed in a 20 μ l volume with 1 μ mol/L forward primer, 1 μ mol/L reverse primer, and 2 μ l of cDNA. Nucleotides, MgCl₂, Taq polymerase, and SYBR Green were included in the SYBR Green PCR master mix (Applied Biosystems, Warrington, UK). Additional SYBR Green (Fisher Biotech, Perth, Australia) was added at 1:1000 dilution. Each sample was amplified in triplicate. The PCR conditions included an incubation at 50°C for 5 minutes to activate uracil-N-glycosylase (UNGase), which removes any carry-over contamination from previous PCR products, followed by a denaturation step at 94°C for 10 minutes. Amplification was carried out for 35 cycles (denaturation at 94°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 30 seconds). Detection of the fluorescent product was measured at the end of the 72°C annealing step. Cycling was

followed by a melt curve analysis (50–90°C with a heating rate of 1° per second) to confirm the amplification of specific PCR products (either procollagen $\alpha_1(I)$ or β -actin), and the absence of non-specific amplification or primer dimer. Primer sequences used for analysis of β -actin and procollagen $\alpha_1(I)$ were as follows: β -actin forward ACT ATC GCC AAT GAG CGG TTC; β -actin reverse ATG CCA CAG GAT TCC ATA CCC; procollagen $\alpha_1(I)$ forward TCG ATT CAC CTA CAG CAC GC; procollagen $\alpha_1(I)$ reverse GAC TGT CTT GCC CCA AGT TCC. Primers were designed using Primer Express software (PerkinElmer Life Sciences, Boston, MA). Results for each sample are calculated as a percentage of the β -actin concentration and compared to that of "control" untreated HSC and expressed as fold-increase of the "control" expression.

Statistical Analysis

Results for all experiments represent duplicate, triplicate, or quadruplicate determinations of between two and four experiments from separate HSC isolations. Statistical significance was calculated using Student's *t*-test, and a *P* value of <0.05 was considered significant. Results are represented as means \pm SE.

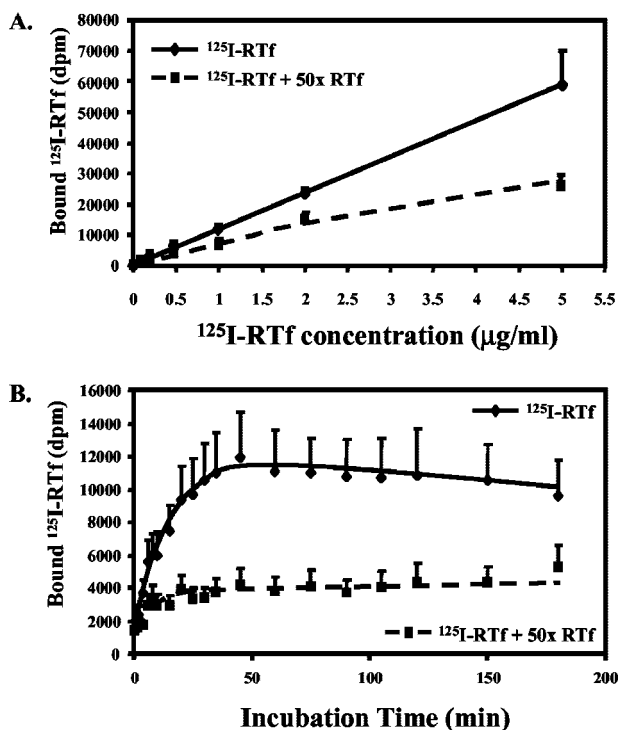


Figure 1. A: Optimization of ¹²⁵I-RTf concentration. HSC were incubated with increasing concentrations of ¹²⁵I-RTf \pm 50-fold excess of unlabelled RTf at 37°C for 2 hours. Optimal specific binding was achieved using 0.5 μ g/ml ¹²⁵I-RTf (59.9 \pm 7.3%). Mean \pm SE, *n* = 2, DPM = disintegrations per minute. **B:** Time course of ¹²⁵I-RTf uptake by HSC. HSC were incubated with 0.5 μ g/ml ¹²⁵I-RTf \pm 50-fold excess of unlabelled RTf at 37°C for up to 180 minutes. Binding reached saturation at \sim 45 minutes. Mean \pm SE, *n* = 3. DPM, disintegrations per minute.

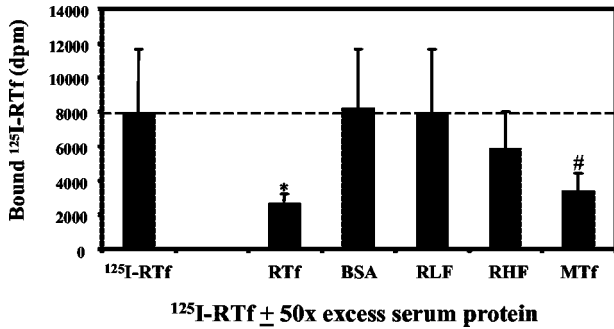


Figure 2. Identification of the HSC transferrin receptor. HSC were incubated with 0.5 $\mu\text{g/ml}$ ^{125}I -RTf \pm 50-fold excess of either unlabelled RTf, BSA, RLF, RHF or MTF at 37°C for 2 hours. HSC demonstrated $67.4 \pm 19.3\%$ specific binding for RTf. Mean \pm SE, $n = 3$, * $P = 0.01$, # $P = 0.02$ when compared to ^{125}I -RTf. DPM, disintegrations per minute. MTF, mouse transferrin; BSA, bovine serum albumin; RLF, rat liver ferritin; RHF, rat heart ferritin.

Results

Optimization of ^{125}I -RTf Concentration and Time Course Study

HSC were incubated with increasing concentrations of ^{125}I -RTf for 120 minutes. There was an increase in the level of ^{125}I -RTf binding by HSC. The level of specific binding (as determined by competitive displacement assays) increased to $59.9\% \pm 7.3\%$ using ^{125}I -RTf at a concentration of 0.5 $\mu\text{g/ml}$ (Figure 1A). The level of specific binding did not increase at higher ^{125}I -RTf concentrations. ^{125}I -RTf at 0.5 $\mu\text{g/ml}$ was then used for all following uptake experiments. After incubation of HSC with 0.5 $\mu\text{g/ml}$ ^{125}I -RTf for increasing times from 1 to 180 minutes there was a rapid increase in ^{125}I -RTf binding which reached a maximum at approximately 45 minutes (Figure 1B).

Competitive Binding Assay

Specificity for RTf

Transferrin receptor binding was demonstrated on HSC cultured for 5 days by competitive displacement of

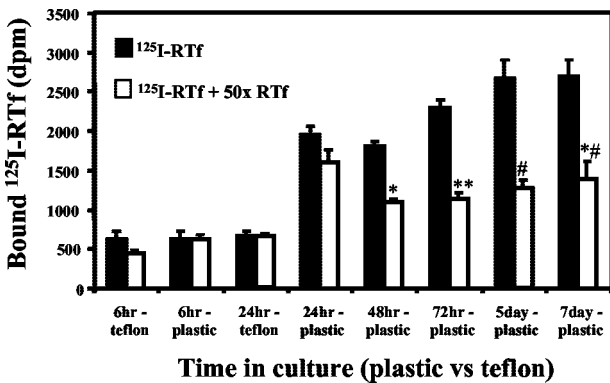


Figure 3. Competitive binding assay in quiescent and activated HSC. HSC were incubated at 37°C for 2 hours with 0.5 $\mu\text{g/ml}$ ^{125}I -RTf \pm 50-fold excess of unlabelled RTf. Cells were cultured on Teflon inserts (to maintain HSC in a quiescent phenotype) and on tissue culture plastic (to activate HSC). ^{125}I -RTf uptake was examined at 6, 24, 48, and 72 hours, and 5 and 7 days post-isolation. Quiescent HSC did not express Tf receptors; however, those cultured on plastic, and therefore culture-activated, did demonstrate competitive displacement of ^{125}I -RTf by a 50-fold excess of unlabelled Tf. Mean \pm SE, $n = 3$, * $P = 0.007$, ** $P = 0.003$, # $P = 0.01$, ## $P = 0.05$, when compared to ^{125}I -RTf. DPM, disintegrations per minute.

^{125}I -RTf by a 50-fold excess of unlabelled RTf. Specific binding of ^{125}I -RTf to HSC was $67.4\% \pm 19.3$, $P = 0.01$ (Figure 2). There was no significant competitive displacement of ^{125}I -RTf by bovine serum albumin, rat heart ferritin, or rat liver ferritin; however, there was significant displacement by mouse transferrin (specific binding $58.6\% \pm 14.4$, $P = 0.02$).

Evidence for a TfR on Quiescent versus Activated HSC

To determine whether binding for RTf by TfR was present on quiescent versus activated HSC, competitive displacement assays were performed on both cell populations. At 6 hours post-isolation, HSC did not demonstrate specific binding of RTf. There was no competitive displacement of ^{125}I -RTf by a 50-fold excess of unlabeled RTf by HSC cultured on either Teflon inserts or cells plated directly onto tissue culture plastic. By 24 hours post-isolation, cells cultured on Teflon demonstrated no specific binding of ^{125}I -RTf. HSC cultured on plastic for 24 hours post-isolation demonstrated specific binding of 18%. By 48 hours, 72 hours, 5 days, and 7 days there was significant displacement of ^{125}I -RTf by a 50-fold excess of unlabelled RTf with specific binding of 38.8%, 50.3%, 52%, and 48.5%, respectively ($P = 0.007$, 0.003, 0.01, and 0.05, respectively; Figure 3).

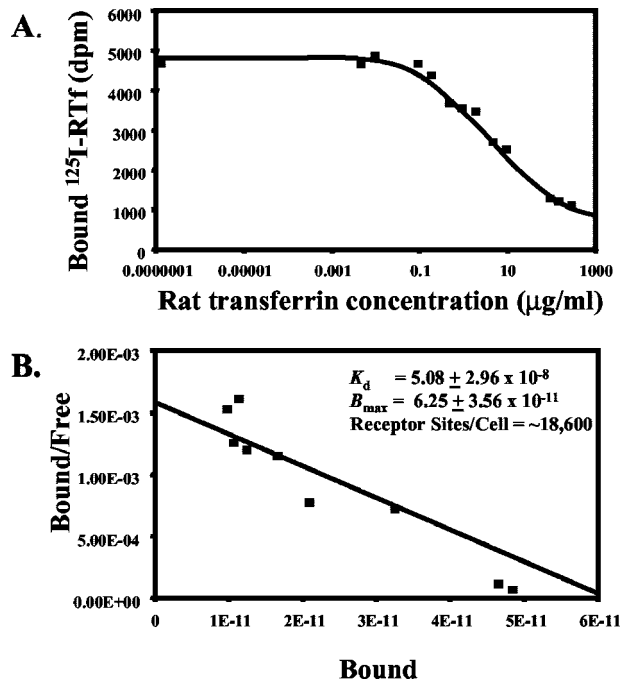


Figure 4. Scatchard analysis of the competitive displacement of ^{125}I -RTf by unlabeled RTf by the HSC transferrin receptor. **A:** HSC were incubated with 0.5 $\mu\text{g/ml}$ ^{125}I -RTf \pm increasing concentrations of unlabelled RTf at 37°C for 2 hours. **B:** Scatchard analysis indicated a single class of binding sites for RTf with an estimated K_d of $5.08 \pm 2.96 \times 10^{-8}$ and a B_{max} of $6.25 \pm 3.56 \times 10^{-11}$, with approximately 18,600 receptor sites per cell. Results are expressed as means from duplicate determinations in each experiment from HSC isolated from two separate rats.

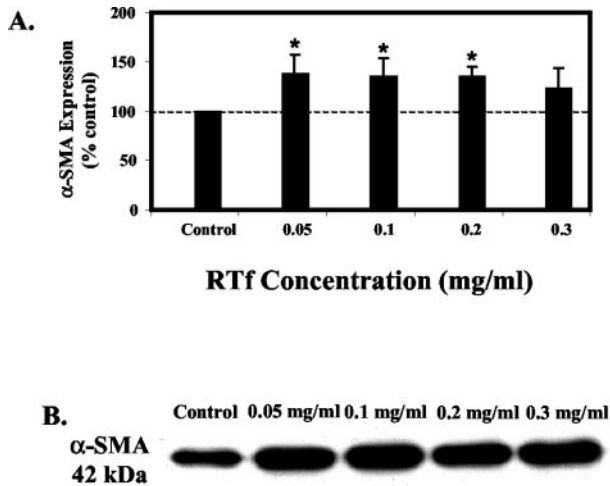


Figure 5. Expression of α -SMA in HSC. **A:** HSC were treated with increasing concentrations of RTf and collected after 7 days in culture and quantitated using scanning laser densitometry following Western blotting. **(B)** Representative Western blot of HSC cell extracts (10 μ g cell protein/lane) showing 42 kd band corresponding to α -SMA. Results are mean \pm SE, $n = 4$. * $P < 0.05$ when compared to control.

Scatchard Analysis

Scatchard analysis of the competitive displacement of 125 I-RTf by unlabelled RTf (Figure 4A) suggested a single class of binding sites for RTf on HSC with an estimated K_d of $5.08 \pm 2.96 \times 10^{-8}$ mol/L and a maximum binding capacity (B_{max}) of $6.25 \pm 3.56 \times 10^{-11}$ mol/L (Figure 4B). Rat HSC express approximately $18,600 \pm 7602$ (mean \pm SE; range, 11,200 – 26,400) receptor sites per cell.

Effect of Transferrin on HSC Activation

α -SMA Expression

RTf caused a significant 35% to 40% increase in α -SMA expression (Figure 5) using 0.05, 0.1, and 0.2 mg/ml RTf. RTf used at a concentration of 0.3 mg/ml also caused an increase in α -SMA expression of approximately 20%; however, this did not reach statistical significance.

HSC Proliferation

RTf significantly reduced HSC proliferation, as determined by 3 H]thymidine incorporation. Proliferation decreased by approximately 45% to 66% at RTf concentrations ranging from 0.05 to 0.5 mg/ml (Figure 6).

Procollagen $\alpha_1(I)$ Gene Expression

Freshly isolated HSC treated with RTf showed no significant difference in procollagen $\alpha_1(I)$ gene expression compared to controls for up to 3 days post-isolation (results not shown). When 5-day culture-activated HSC were treated with RTf for 6 hours, there was an increase in procollagen $\alpha_1(I)$ mRNA expression compared to untreated HSC (1.8-fold versus controls); however, this did not reach statistical significance ($P = 0.17$). After treatment for 48 hours there was a significant increase in

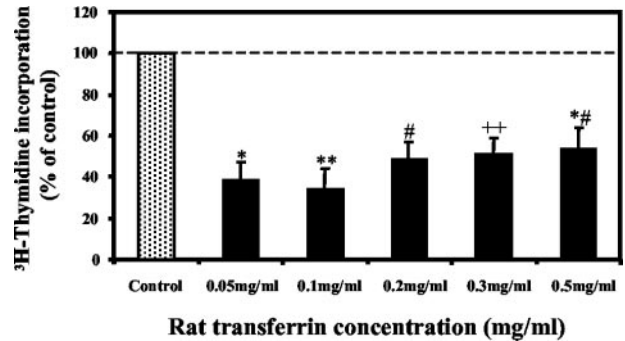


Figure 6. Effect of RTf on HSC proliferation as determined by 3 H]thymidine incorporation. Results are expressed as a percentage of control and calculated as disintegrations/min. Results are mean \pm SE of quadruplicate determinations in each experiment from HSC isolated from two separate rats. * $P = 0.002$, ** $P = 0.001$, # $P = 0.009$, ## $P = 0.01$, *# $P = 0.03$, when compared to controls.

procollagen expression (1.5-fold versus controls, $P = 0.005$) (Figure 7).

Discussion

These studies are the first to demonstrate the presence of a specific, high-affinity receptor for transferrin on culture-activated HSC. Scatchard analysis demonstrated a single class of binding sites with a K_d of $5.08 \pm 2.96 \times 10^{-8}$ mol/L, a maximum binding capacity of $6.25 \pm 3.56 \times 10^{-11}$ mol/L with approximately 18,600 receptor sites per cell. The binding of transferrin to its receptor reached a maximum at approximately 45 minutes with the greatest specific binding ($67.4 \pm 19.3\%$) observed using 0.5 μ g/ml 125 I-RTf. In addition, this study has provided important new information which shows that transferrin plays a role in regulating the activated phenotype of HSC, as demonstrated by the transferrin-induced increase in

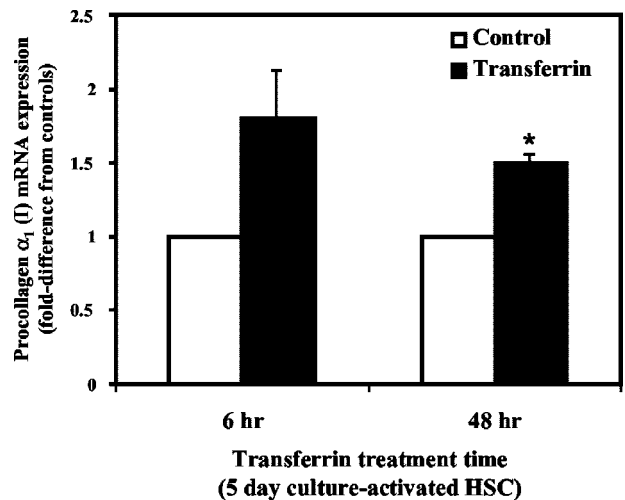


Figure 7. Effect of RTf on HSC procollagen $\alpha_1(I)$ mRNA expression as determined by real-time RT-PCR analysis. Day 5 HSC were treated with 0.2 mg/ml RTf. RNA was collected at 6 and 48 hours after treatment with RTf. Procollagen $\alpha_1(I)$ mRNA expression in RTf-treated HSC are expressed as a fold-difference from controls (untreated HSC), after standardization against expression of the housekeeping gene β -actin. * $P = 0.005$. Mean \pm SE, $n = 3$.

α -SMA and procollagen $\alpha_1(I)$ mRNA expression, and decreased HSC proliferation.

This study has demonstrated that the HSC TfR is specific for transferrin, as a 50-fold excess of unlabeled rat transferrin was able to significantly inhibit binding of ^{125}I -RTf. Mouse transferrin was also able to inhibit binding of ^{125}I -RTf. This kind of cross-species specificity of TfR for transferrin has previously been demonstrated in other cell types by Lim et al,²² who showed that uptake of heterologous human or rabbit transferrin by rat reticulocytes was actually in excess of that for rat transferrin (150% and 200% of control, respectively). Uptake of transferrin by rabbit reticulocytes has previously been shown to be a temperature-dependent process, which reaches a steady state by approximately 20 minutes of incubation.²³ We have assessed transferrin binding in HSC and have demonstrated maximal binding of transferrin after approximately 45 minutes of incubation at 37°C.

One of the aims of this study was to determine whether TfR were present on both quiescent and activated HSC. The fact that these receptors are not present on quiescent cells is of interest when attempting to understand the mechanisms of iron-induced HSC activation. This observation may be a reflection of the reduced iron requirements of the quiescent HSC phenotype. Most cells take up iron by predominantly receptor-mediated means,¹² but hepatocytes in culture also take up transferrin via a non-receptor-mediated pathway.²⁴ Quiescent HSC, which may have lower iron requirements than activated HSC, may also use alternative pathways of iron uptake, although further investigation is required to elucidate these pathways. A second transferrin receptor (TfR2) has been recently described,²⁵ which raises the possibility that this receptor may play a role in HSC iron uptake. However, we have examined TfR2 mRNA expression in HSC by RNase protection assay and have found no expression of this receptor in either quiescent or activated HSC (unpublished observations).

Transferrin receptors have been characterized on a number of cell types including hepatocytes, Kupffer cells, reticulocytes, fibroblasts, neuronal cells and various cell lines.^{7,26-29} Previous reports have estimated that the TfR is a high affinity binding site for transferrin with a K_d ranging from 1.65×10^{-7} to 27×10^{-9} mol/L. We have provided the first evidence for a specific, high-affinity receptor for Tf on activated HSC with a K_d of $5.08 \pm 2.96 \times 10^{-8}$ mol/L.

While the identification and characterization of the HSC TfR are novel, the role of the TfR in HSC iron metabolism or in the transformation into myofibroblasts is unclear. It is postulated that the TfR may function to supply the activated HSC with its increasing requirement for iron. As HSC sequester little iron in comparison to hepatocytes and Kupffer cells in conditions of iron overload, it is unlikely that the HSC TfR plays a major role in the release of iron for storage within ferritin, as is the case in other cells.³⁰ Iron may rapidly transit intracellular iron pools before being used by the cell for the synthesis of iron-requiring enzymes and proteins. Ramm and colleagues¹⁰ have previously demonstrated that activated HSC internalize iron-loaded tissue ferritin. It is postulated that iron de-

rived from the phagocytosis of senescent red blood cells by Kupffer cells is released as ferritin,³¹ which can then be taken up by hepatic cells possessing ferritin receptors, such as hepatocytes³² and HSC,¹⁰ in the paracellular movement of iron in the liver.^{10,32,33} Thus, it is possible that HSC may play a role in the transit (via transferrin) and storage (via ferritin) of iron in liver iron metabolism in conjunction with other hepatic cells such as hepatocytes and Kupffer cells, although the precise physiological nature of these pathways remain unknown.

As activated rather than quiescent HSC possess receptors for transferrin it is also possible that the interaction of RTf with the HSC TfR plays a role in the regulation of iron-induced activation of HSC. This is the first study to demonstrate that RTf causes a significant increase in HSC activation, as determined by western blotting for α -SMA. In contrast, studies by Ramm et al²¹ demonstrated that long-term exposure to rat liver ferritin caused a dose-dependent decrease in α -SMA expression by HSC over the same time course. While this group initially suggested that this effect of ferritin may be induced by iron, further studies suggest that long-term exposure to ferritin may act to inhibit protein kinase C, which may control α -SMA expression *via* cell signaling pathways.³⁴ While it would be of interest to examine the effects of iron-loaded transferrin versus iron-depleted transferrin (apotransferrin), to delineate the role of iron delivery to HSC, inherent problems exist with this approach, because apotransferrin is not recognized by the TfR.³⁵ Further studies are required to examine the mechanisms associated with the RTf-induced increase in α -SMA expression.

RTf caused a significant decrease in HSC proliferation as determined by [3H]thymidine incorporation. These observations appear to contradict the α -SMA results, as with an increased HSC activation the proliferative capacity of HSC might have been expected to increase. Transferrin has previously been shown to increase proliferation of a number of cell types, including pituitary tumor cells,³⁶ B-cells³⁷ and cultured blastemal cells.³⁸ In contrast, Hagiwara et al³⁹ have shown that iron-free transferrin is not growth-stimulating for a rat intestinal epithelial cell line, and Booth et al⁴⁰ failed to demonstrate a significant effect of transferrin on mouse colonic epithelium.

RTf did not significantly influence the expression of procollagen $\alpha_1(I)$ mRNA in freshly isolated HSC for up to 3 days post-treatment. However, there was an increase in procollagen $\alpha_1(I)$ gene expression at 6 and 48 hours post-transferrin treatment in 5-day culture-activated HSC. Interestingly, the effects of transferrin on both proliferation and type I collagen gene expression by HSC show similarities to the effects of transforming growth factor- β (TGF- β) on HSC activation *in vitro*. TGF- β has been shown to both inhibit HSC proliferation and increase procollagen $\alpha_1(I)$ mRNA expression.^{41,42} In addition to its role as an iron transport protein, transferrin may have cytokine-like actions on HSC, similar to those of TGF- β . Indeed it is possible that in HSC-signaling pathways elicited via the interaction of transferrin with its receptor may be similar to those involved in TGF- β signaling. However, further studies are required to fully elucidate the signaling pathways

associated with transferrin-induced expression of the type I collagen gene in activated HSC.

In summary, these studies have identified a specific high-affinity TfR on activated HSC, which is not present in the quiescent HSC phenotype. In addition, this study is the first to demonstrate that transferrin plays a role in regulating HSC activation *in vitro*. Further studies are required to fully clarify the influence of transferrin on HSC activation, particularly in conditions of iron overload *in vivo*.

Acknowledgments

The rat holo-transferrin was a generous gift from Dr. Gregory Anderson, the Queensland Institute of Medical Research, Australia.

References

- Friedman SL: Hepatic stellate cells. *Prog Liver Dis* 1996, 14:101–130
- Ooi LP, Crawford DH, Gotley DC, Clouston AD, Strong RW, Gobe GC, Halliday JW, Bridle KR, Ramm GA: Evidence that "myofibroblast-like" cells are the cellular source of capsular collagen in hepatocellular carcinoma. *J Hepatol* 1997, 26:798–807
- Ramm GA, Nair VG, Bridle KR, Shepherd RW, Crawford DH: Contribution of hepatic parenchymal and nonparenchymal cells to hepatic fibrogenesis in biliary atresia. *Am J Pathol* 1998, 153:527–535
- Ramm GA, Crawford DH, Powell LW, Walker NI, Fletcher LM, Halliday JW: Hepatic stellate cell activation in genetic haemochromatosis. Lobular distribution, effect of increasing hepatic iron and response to phlebotomy. *J Hepatol* 1997, 26:584–592
- Ramm GA, Li SC, Li L, Britton RS, O'Neill R, Kobayashi Y, Bacon BR: Chronic iron overload causes activation of rat lipocytes *in vivo*. *Am J Physiol* 1995, 268:G451–G458
- Pietrangelo A, Gualdi R, Casalgrandi G, Montosi G, Ventura E: Molecular and cellular aspects of iron-induced hepatic cirrhosis in rodents. *J Clin Invest* 1995, 95:1824–1831
- Kumazawa M, Misaki M, Baba M, Shima T, Suzuki S: Transferrin receptor on rat Kupffer cells in primary culture. *Liver* 1986, 6:138–144
- Osterloh K, Aisen P: Pathways in the binding and uptake of ferritin by hepatocytes. *Biochim Biophys Acta* 1989, 1011:40–45
- Trinder D, Batey RG, Morgan EH, Baker E: Effect of cellular iron concentration on iron uptake by hepatocytes. *Am J Physiol* 1990, 259:G611–G617
- Ramm GA, Britton RS, O'Neill R, Bacon BR: Identification and characterization of a receptor for tissue ferritin on activated rat lipocytes. *J Clin Invest* 1994, 94:9–15
- Worwood M: An overview of iron metabolism at a molecular level. *J Intern Med* 1989, 226:381–391
- Aisen P: Transferrin, the transferrin receptor, and the uptake of iron by cells. *Met Ions Biol Syst* 1998, 35:585–631
- Page MA, Baker E, Morgan EH: Transferrin and iron uptake by rat hepatocytes in culture. *Am J Physiol* 1984, 246:G26–G33
- Ramm GA, Britton RS, O'Neill R, Blaner WS, Bacon BR: Vitamin A-poor lipocytes: a novel desmin-negative lipocyte subpopulation, which can be activated to myofibroblasts. *Am J Physiol* 1995, 269:G532–G541
- Halliday JW, Powell LW, Mack U: Iron absorption in the rat: the search for possible intestinal mucosal carriers. *Br J Haematol* 1976, 34:237–250
- Bolton AE, Hunter WM: The labelling of proteins to high specific radioactivities by conjugation to a 125I-containing acylating agent. *Biochem J* 1973, 133:529–539
- Kishimoto T, Tavassoli M: Recovery of transferrin receptors on hepatocytes membrane after collagenase perfusion. *Biochem Biophys Res Commun* 1986, 134:711–715
- Munson PJ, Rodbard D: Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 1980, 107:220–239
- Rockey DC, Boyles JK, Gabbiani G, Friedman SL: Rat hepatic lipocytes express smooth muscle actin upon activation *in vivo* and in culture. *J Submicrosc Cytol Pathol* 1992, 24:193–203
- Peterson GL: A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 1977, 83:346–356
- Ramm GA, Britton RS, O'Neill R, Kohn HD, Bacon BR: Rat liver ferritin selectively inhibits expression of α -smooth muscle actin in cultured rat lipocytes. *Am J Physiol* 1996, 270:G370–G375
- Lim BC, McArdle HJ, Morgan EH: Transferrin-receptor interaction and iron uptake by reticulocytes of vertebrate animals—a comparative study. *J Comp Physiol B* 1987, 157:363–371
- Iacopetta BJ, Morgan EH: The kinetics of transferrin endocytosis and iron uptake from transferrin in rabbit reticulocytes. *J Biol Chem* 1983, 258:9108–9115
- Trinder D, Morgan EH, Baker E: The effects of an antibody to the rat transferrin receptor and of rat serum albumin on the uptake of diferric transferrin by rat hepatocytes. *Biochim Biophys Acta* 1988, 943:440–446
- Kawabata H, Yang R, Hiramata T, Vuong PT, Kawano S, Gombart AF, Koeffler HP: Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. *J Biol Chem* 1999, 274:20826–20832
- Ward JH, Kushner JP, Kaplan J: Regulation of HeLa cell transferrin receptors. *J Biol Chem* 1982, 257:10317–10323
- Grasso JA, Bruno M, Yates AA, Wei LT, Epstein PM: Calmodulin dependence of transferrin receptor recycling in rat reticulocytes. *Biochem J* 1990, 266:261–272
- Graziadei I, Kahler CM, Wiedermann CJ, Vogel W: The acute-phase protein α 1-antitrypsin inhibits transferrin-receptor binding and proliferation of human skin fibroblasts. *Biochim Biophys Acta* 1998, 1401:170–176
- Roskams AJ, Connor JR: Transferrin receptor expression in myelin deficient (md) rats. *J Neurosci Res* 1992, 31:421–427
- Halliday JW, Ramm GA, Powell LW: Cellular iron processing and storage: The role of ferritin. *Iron Metabolism in Health and Disease*. Edited by JH Brock, JW Halliday, MJ Pippard, LW Powell. London, Academic Press/United Kingdom, 1994, pp 97–122
- Kondo H, Saito K, Grasso JP, Aisen P: Iron metabolism in the erythrophagocytosing Kupffer cell. *Hepatology* 1988, 8:32–38
- Sibille JC, Kondo H, Aisen P: Interactions between isolated hepatocytes and Kupffer cells in iron metabolism: a possible role for ferritin as an iron carrier protein. *Hepatology* 1988, 8:296–301
- Ono T, Seno S: Transport of ferritin from Kupffer cells to liver parenchymal cells. Morphological and immunocytochemical observations. *Int J Hematol* 1991, 54:93–102
- Ramm GA, Piva TJ, Nair VG, Garrone BM, Watters DJ, Crawford DHG: Role of tissue ferritin in regulation of α -smooth muscle actin expression in hepatic stellate cells via PKC ζ and/or nitric oxide. *Hepatology* 1997, 26:451A (abstract)
- Morgan EH: Effect of pH and iron content of transferrin on its binding to reticulocyte receptors. *Biochim Biophys Acta* 1983, 762:498–502
- Tampanaru-Sarmesiu A, Stefanescu L, Thapar K, Kontogeorgos G, Sumi T, Kovacs K: Transferrin and transferrin receptor in human hypophysis and pituitary adenomas. *Am J Pathol* 1998, 152:413–422
- Bosseloir A, Bouzahzah F, Defrance TH, Heinen E, Simar LJ: The influence of follicular dendritic cells on B-cell proliferation depends on the activation of B cells and the mitogen used. *Scand J Immunol* 1996, 43:23–30
- Mescher AL, Connell E, Hsu C, Patel C, Overton B: Transferrin is necessary and sufficient for the neural effect on growth in amphibian limb regeneration blastemas. *Dev Growth Differ* 1997, 39:677–684
- Hagiwara T, Shinoda I, Fukuwatari Y, Shimamura S: Effects of lactoferrin and its peptides on proliferation of rat intestinal epithelial cell line, IEC-18, in the presence of epidermal growth factor. *Biochem Biotechnol Biochem* 1995, 59:1875–1881
- Booth C, Patel S, Bennion GR, Potten CS: The isolation and culture of adult mouse colonic epithelium. *Epithelial Cell Biol* 1995, 4:76–86
- Saile B, Matthes N, Knittel T, Ramadori G: Transforming growth factor β and tumor necrosis factor α inhibit both apoptosis and proliferation of activated rat hepatic stellate cells. *Hepatology* 1999, 30:196–202
- Pinzani M: Novel insights into the biology and physiology of the Ito cell. *Pharmacol Ther* 1995, 66:387–412