Effects of Cell Proliferation on the Uptake of Transferrin-Bound Iron by Human Hepatoma Cells

Adrian W. M. Lee,1 Phillip S. Oates,1 and Deborah Trinder2,3

The effects of cellular proliferation on the uptake of transferrin-bound iron (Tf-Fe) and expression of transferrin receptor-1 (TfR1) and transferrin receptor-2 (TfR2) were investigated using a human hepatoma (HuH7) cell line stably transfected with TfR1 antisense RNA expression vector to suppress TfR1 expression. At transferrin (Tf) concentrations of 50 nmol/L and 5 μmol/L, when Tf-Fe uptake occurs by the TfR1- and TfR2-independent (NTfR1)-mediated process, respectively, the rate of Fe uptake by proliferating cells was approximately 250% that of stationary cells. The maximum rate of Fe uptake by the TfR1- and NTfR1-mediated process by proliferating cells was increased to 200% and 300% that of stationary cells, respectively. The maximum binding of Tf by both TfR1- and NTfR1-mediated processes by proliferating cells was increased significantly to 160% that of stationary cells. TfR1 and TfR2-α protein levels expressed by proliferating cells was observed to be approximately 300% and 200% greater than the stationary cells, respectively. During the proliferating growth phase, expression of TfR1 messenger RNA (mRNA) increased to 300% whereas TfR2-α mRNA decreased to 50% that of stationary cells. In conclusion, an increase in Tf-Fe uptake by TfR1-mediated pathway by proliferating cells was associated with increased TfR1 mRNA and protein expression. An increase in Tf-Fe uptake by NTfR1-mediated pathway was correlated with an increase in TfR2-α protein expression but not TfR2-α mRNA. In conclusion, TfR2-α protein is likely to have a role in the mediation of Tf-Fe uptake by the NTfR1 process by HuH7 hepatoma cell in proliferating and stationary stages of growth. (HEPATOLOGY 2003;38:967-977.)

Iron is essential for many biological functions, but, in excess, free radical production increases and organ damage follows.1 Under normal conditions, transferrin (Tf) tightly binds plasma Fe. Excess Fe is taken up from the circulation and stored mainly by the liver within hepatocytes.2 Thus, the hepatocyte has developed efficient mechanisms for the uptake and assimilation of Tf-bound iron (Tf-Fe).3,4 These involve uptake of Tf-Fe by the Tf receptor-1 (TfR1)-mediated process and another mechanism independent of the Tf receptor-1 (NTfR1).5 The TfR1-mediated process involves endocytosis of Tf-Fe into the cell through membrane-bound TfR1.6,7 The binding affinity of diferric Tf to TfR1 at an extracellular pH of 7.4 is high with an estimated dissociation constant of 2 to 7 nmol/L.6,7 Because plasma diferric Tf circulates at a concentration above 5 μmol/L,8 and TfR1 saturates in the nanomolar range, TfR1 will remain saturated with Tf-Fe. The binding affinity of diferric Tf to TfR1 at an extracellular pH of 7.4 is high with an estimated dissociation constant of 2 to 7 nmol/L.6,7 Because plasma diferric Tf circulates at a concentration above 5 μmol/L,8 and TfR1 saturates in the nanomolar range, TfR1 will remain saturated with Tf-Fe.7 Tf-Fe-TfR1 complex is internalized into low-density vesicles followed by the pH-dependent release of Fe from the complex. The Fe is then transported across the endosomal membrane by the divalent metal transporter-1.9 Once in the cytosol, Fe is used for the synthesis of heme proteins by the mitochondria or stored as ferritin. The apo-Tf-TfR1 complex is recycled to the cell surface, where at a neutral pH apo-Tf dissociates from its receptor.

The regulation of expression of TfR1 in hepatocytes has been investigated intensively under a variety of con-
itions, such as cellular proliferation, with Fe status and in the presence of cytokines. Cellular proliferation after partial hepatectomy increased TfR1 expression. Similarly, Fe deficiency increased TfR1 expression in primary adult rat hepatocyte cultures. Conversely, Fe loading of primary hepatocyte cultures decreased the number of TfR1. Cytokines such as interleukin 1β, interleukin 6, and tumor necrosis factor α increased TfR1 numbers and uptake of Tf-Fe in the human hepatoblastoma cell line, HepG2. In view of this, it is clear that TfR1 expression is altered under a variety of physiological conditions, such as proliferation, cellular Fe status, and inflammation.

Recently, a gene with homology to the TfR1 has been cloned and called Tf receptor 2 (TfR2). In the liver, it is abundantly expressed from a single gene that undergoes alternative splicing to express in either TfR2-α (2.9kb) or TfR2-β (2.5kb) transcripts. The TfR2-α has high homology to TfR1 in its extracellular-ligand binding domain, whereas TfR2-β lacks the transmembrane and cytosolic domains, suggesting that it may be a soluble cytosolic receptor. When TfR2-α is overexpressed in Chinese Hamster Ovary (CHO) cells, there was an increase in Tf binding and Fe uptake. The affinity of TfR2-α for Tf was 30 times lower than TfR1 for Tf. Therefore, 30 times more Tf is required to saturate TfR2-α than TfR1. Given that under normal physiological conditions, plasma Tf levels are above that required to saturate the TfR1 on hepatocyte and hepatoma cells, the uptake of Tf-Fe by the liver is likely to occur primarily by the NTfR1 pathway; however, whether TfR2-α mediates this process is unknown.

Furthermore, in the MG63 osteosarcoma cell line, transcription of TfR1 and TfR2-α was shown to be cell cycle-dependent, suggesting that expression of TfR1 and TfR2-α are coordinately up-regulated by cellular proliferation. However, whether TfR2-α, which is highly expressed by hepatocytes and hepatoma cells, is also up-regulated during proliferation of hepatoma is unclear.

In this study, the effects of cellular proliferation on the uptake of Tf-Fe by the TfR1 and NTfR1-mediated pathways and the role of TfR1 and TfR2-α in these processes were investigated in a HuH7 human hepatoma cell line transfected with a TfR1 antisense RNA expression vector that suppressed TfR1 expression by 50% to 60%. Use of these cells allowed us to clearly differentiate uptake of Tf-Fe by proliferating and stationary hepatoma cells by both the TfR1- and NTfR1-mediated processes.

Materials and Methods

**Hepatoma Cell Line Culture.** A human hepatoma HuH7 cell line stably transfected with human TfR1 antisense RNA expression vector to suppress TfR1 expression was used. The transfected cells were cultured in plastic tissue culture flasks (Nunc, Naperville, IL) in Minimal Essential Media supplemented with 10% fetal calf serum (Invitrogen, Melbourne, Australia), 100 U/mL penicillin (Sigma, St Louis, MO), 100 μg/mL streptomycin (Sigma), 5μg/mL fungizone (Invitrogen), and 200 μg/mL gentamicin (Invitrogen).

**Fe Uptake and Tf Binding by Hepatoma Cells.** Human Tf was labeled with radiolabeled 59Fe and 125I as previously described. For experiments, the cells were plated out into 6-well tissue culture plates containing approximately 2 × 10³ cells/well and grown for 70 and 145 hours to obtain cells in the proliferating and stationary growth phases, respectively. Endogenous Tf and fetal calf serum were removed by washing cells twice with Hanks' buffer pH 7.4 at 37°C. The cells were incubated in 6-well plates with Minimal Essential Media containing 2% bovine serum albumin and 59Fe-125I-Tf either over the concentration range 5 nmol/L to 25 μmol/L for 60 minutes. Fe uptake was measured at 37°C whereas Tf binding was measured at 4°C. Uptake of Fe and Tf with time were measured by incubating the cells with 50 nmol/L or 5 μmol/L 59Fe-125I-Tf for 0 to 90 minutes at 37°C. Then, cells were washed five times with Hanks' buffer and solubilized with 0.1% Triton/0.1N NaOH. Aliquots were counted for radioactivity in a LS6500 p-scintillation counter (Beckman). Protein concentrations were measured by bicinchoninic acid protein assay (Pierce, Rockford, IL).

**Proliferative Status of Cells.** Cells in proliferating and stationary phases were washed twice with Hanks' buffer at 37°C and incubated with 1 μCi/mL [³H]-thymidine in Minimal Essential Media at 37°C for 1 hour. The cells were then washed 4 times with Hanks' buffer at 4°C and incubated with 20% trichloroacetic acid for an hour on ice, then centrifuged and washed with 10% trichloroacetic acid at 4°C. The pellet was dissolved in 1 mol/L NaOH, and Ready Safe scintillant (Beckman, Fullerton, CA) was added to samples and radioactivity measured in a LS6500 β-scintillation counter (Beckman). Experiments were performed concurrently with Tf and Fe uptake and gene/protein expression studies to determine changes in the proliferative status of the cells.

**RNA Analysis.** Partial complementary DNAs (cDNAs) encoding human TfR1, TfR2-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were produced as summarized in Table 1. Templates for amplification of TfR1 was a recombinant plasmid pRC/CMV/TfR123; TfR2-α was a pCDNA3.1 flag-TfR2-α13; and GAPDH was generated by reverse transcription polymerase chain reaction (RT-PCR) using total RNA.
extracted from HuH7 TfR1 antisense cells and forward and reverse primers shown in Table 1. The PCR products of TfR1 and GAPDH were subcloned into pGEM-T-Easy Vector (Promega, Sydney, Australia), transformed, amplified, and then purified.

The cDNAs of TfR1, TfR2-α, and GAPDH were labeled with random, primer-specific incorporation of nucleotides labeled with digoxigenin-11-dUTP (DIG) (Roche, Mannheim, Germany). A DIG-labeled TfR2-α probe was generated directly from the expression plasmid pCDNA3.1 flag-TfR2-a1-lg.

Proliferating and stationary HuH7 cells were grown in 175-cm² culture flasks seeded at 8 × 10⁶ cells/flask for 70 and 145 hours. Total RNA was isolated from the cells by using RNAwiz (Ambion, Austin, TX). For Northern blot experiments, approximately 1 mg of total RNA was obtained and used for poly (A⁺) messenger RNA (mRNA) isolation, according to the manufacturer’s instructions (Promega, Sydney, Australia). Five to 15 μg of mRNA was loaded onto a denaturing formaldehyde agarose gel and electrophoresed at 15 mA at 4°C overnight. The RNA was transferred onto a nylon membrane (Hybond N⁺) by capillary elution overnight. The membranes were then washed in 6XSSC for 5 minutes, ultraviolet (UV) cross-linked for 5 minutes, and baked for 1 hour at 80°C. Membranes were prehybridized for 30 minutes in ULTRAhyb (Ambion), then hybridized with DIG-labeled probes at 48°C overnight according to the manufacturer’s specifications. The membranes were washed twice at 48°C in 2XSSC, 0.1% sodium dodecyl sulphate (SDS) for 5 minutes followed by 2 washes in 0.1XSSC, 0.1% SDS for 15 minutes. The DIG-labeled cDNA probes were detected, after hybridization to the target RNA, by enzyme-linked immunoassay using an anti-DIG antibody linked to alkaline phosphatase (1:5,000; Roche). Gene expression was detected with 5-bromo-4-chloro-3-indoyl phosphate and nitro-blue tetrazolium salt.

To confirm TfR2-α mRNA expression, semiquantitative PCR was performed using conditions specified in Table 1. Random primed first-strand cDNA was generated using an AMV-RT kit (Roche, Indianapolis, IN) and using 1 μg total RNA as a template. TfR2-α mRNA expression was normalized against GAPDH mRNA expression. Densitometry was performed with National Institutes of Health Image 1.61.

**Western Blot Analysis.** Cells in proliferating and stationary growth phases were lysed in phosphate-buffered saline (pH 7.4) containing 25 mmol/L Tris-Cl, 0.5% Triton X-100 and protease inhibitors, 200 mmol/L phenyl-methylsulfonyl fluoride, 1X Tranyol for 5 minutes at 37°C. The cell suspension was centrifuged at 13,000 rpm to remove insoluble material. Between 25 and 100 μg of protein was separated on a 12% SDS-polyacrylamide gel electrophoresis followed by transfer to an Immobilon-P membrane (Millipore, Bedford, MA). Detection of the specific proteins was performed with specific antisera, and the blots were incubated with an appropriate secondary antibody. Immunoblot analysis of TfR1 was performed with goat anti-human TfR1 antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated rabbit anti-goat (1:1,000) and goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2,000; Serotech, Oxford, United Kingdom). The TfR2 antibody has been shown to be specific for TfR2. This was shown by reaction with CHO cells overexpressing TfR2 but not CHO cells overexpressing TfR1 by immunocytchemistry²⁴ and Western blot (personal communication, H. Kawabata et al., December 2002). Ferritin was detected with a rabbit anti-human ferritin antibody (1:2,000; DAKO) and goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2,000; Serotech). Immunological detected proteins were visualized using an

---

**Table 1. Oligonucleotides Used as Primers for PCR**

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Gene Bank Acc. No.</th>
<th>Technique Used</th>
<th>Position of Sequence</th>
<th>Forward Primer 5'→3'</th>
<th>Reverse Primer 5'→3'</th>
<th>Cycle Conditions</th>
<th>Annealing</th>
<th>Extension</th>
<th>Cycle No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human transferrin receptor 1</td>
<td>M11507</td>
<td>N</td>
<td>1639-2421</td>
<td>ATCCGGTACTGGGGAATT</td>
<td>TCCAGACTACCTGGTGGTCTC</td>
<td>95°C/60 s 55°C/60 s 68°C/90 s</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human transferrin receptor 2</td>
<td>AF067864</td>
<td>N</td>
<td>35-446 (α)</td>
<td>GGGCTGTTGGGCTTATT</td>
<td>AGGTCGCCACAGTAGAG</td>
<td>94°C/30 s 40°C/60 s 68°C/45 s</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>324-274 (α,β)</td>
<td>GGGGCTACGTCGCCCTCC</td>
<td>TGGAGACCTGAAGGCTCCCA</td>
<td>94°C/30 s 55°C/60 s 72°C/40 s</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NM_003227</td>
<td>SQ</td>
<td>355-755 (α)</td>
<td>GGGGCTACGTCGCCCTCC</td>
<td>TGGAGACCTGAAGGCTCCCA</td>
<td>94°C/60 s 60°C/60 s 72°C/60 s</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>XM_006959</td>
<td>N</td>
<td>31-165</td>
<td>GCTCCTCTGACTGGCG</td>
<td>AGTGAGTGGGGAGGCTGAC</td>
<td>94°C/30 s 55°C/60 s 68°C/105 s</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NM_002046</td>
<td>SQ</td>
<td>441-877</td>
<td>GGTCCGCTCTGAGTAGG</td>
<td>CTCCTGCGCTGCACCA</td>
<td>94°C/60 s 65°C/60 s 72°C/60 s</td>
<td>18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Semiquantitative PCR included an extra step at 94°C for 2 minutes before denaturation step.

Abbreviations: N, Northern; SQ, semiquantitative PCR.
ECL Western blotting system (Amersham, Melbourne, Australia) and exposure to Hyperfilm ECL for an optimum exposure time. The exposure time was varied from 15 seconds to 15 minutes to ensure that the substrate reaction was in a linear range over time. The relative amounts of proteins were assessed by densitometry with National Institutes of Health Image 1.61.

Statistical Analysis. The uptake of Fe and Tf is expressed as pmoles of Fe or Tf per mg of protein. All determinations were measured in triplicate within each experiment and the results are expressed as mean ± SE. All experiments were performed 3 to 5 times. Graphical and experimental data were determined with the program PRISM (GraphPad Software, San Diego, CA). Experimental data were compared using the Student’s t-test, and results were considered statistically significant when P < .05.

Results

Cell Growth. HuH7 cells transfected with a TfR1 antisense RNA expression vector that suppressed TfR1 expression by 50% to 60% were used in all experiments. The rate of DNA synthesis was 2- to 3-fold higher by proliferating cells compared with the stationary cells as determined by [3H]-thymidine incorporation.

Uptake of Tf-Fe by Proliferating and Stationary Cells. In both proliferating and stationary cells, uptake of Fe after 1 hour of incubation at 37°C increased with increasing extracellular Tf-Fe concentrations in a biphasic manner (Fig. 1A and B). Analysis of the Fe uptake curves by the Michaelis-Menten equation showed the presence of 2 saturable processes. For both proliferating and stationary cells, the first component saturated at approximately 50 nmol/L Tf, and both had similar Michaelis-Menten constants (Km). However, the maximum rate of Fe uptake (Vmax) by the proliferating and stationary cells differed. For proliferating cells the Vmax was 37.1 ± 4.8 pmoles Fe/mg protein/60 min and for the stationary cells the Vmax was 19.6 ± 2.4 pmoles Fe/mg protein/60 min (mean ± SE; n = 4). Therefore, the Vmax for the proliferating cells was approximately 2-fold greater than for stationary cells, and the difference was significant at P < .05. In both cellular conditions, the second component saturated at concentrations above 10 μmol/L Tf-Fe and had similar Km (Fig. 1B). The Vmax for proliferating cells was 167.9 ± 38.2 pmoles Fe/mg protein/60 min and for stationary cells was 49.3 ± 4.7 pmoles Fe/mg protein/60 min (mean ± SE; n = 4). Therefore, proliferating cells took up more than 3-fold more Fe than the stationary cells. This difference was significant (P < .05).

Tf Binding by Proliferating and Stationary Cells. The binding of Tf by the proliferating and stationary cells after 1 hour incubation at 4°C increased with increasing Tf concentrations in a biphasic manner (Fig. 2A and B). Analysis of Tf binding by the Scatchard method showed 2 saturable components indicating the presence of 2 types of Tf binding sites. For both stages of cell growth, the first component reached saturation at approximately 50 nmol/L Tf (Fig. 2A). The maximum binding of Tf (Bmax) by proliferating cells was significantly increased (P < .05) compared with stationary cells. The Bmax values for proliferating cells and stationary cells were 2.1 ± 0.2 pmoles Tf/mg protein (mean ± SE; n = 5) and 1.3 ± 0.1 pmoles Tf/mg protein (mean ± SE; n = 5), respectively, and no differences observed for the affinity constant (Ks).

The second saturable component reached saturation at approximately 10 μmol/L Tf (Fig. 2B) with Bmax values for proliferating cells (17.8 ± 1.9 pmoles Tf/mg protein;
TF binding from 50 nmol/L Tf by proliferating and stationary cells reached a steady state after approximately 30 minutes. The steady-state level of TF binding by the proliferating cells was significantly increased to approxi-

mean ± SE; n = 5) significantly increased 1.6-fold compared with the stationary cells (11.4 ± 1.4 pmol TF/mg protein, mean ± SE; n = 5). No difference was observed for the Ka value for cells in different stages of cell growth.

**Effect of Cellular Proliferation on TF-Fe Uptake With Time.** The uptake of TF-Fe by proliferating and stationary cells was investigated by incubating with 50 nmol/L Tf, where TF-Fe uptake occurred primarily by the TR1-mediated process and 5 μmol/L Tf where TF-Fe uptake occurred mainly by the NTfR1-mediated process (Figs. 3 and 4). Fe uptake by proliferating and stationary cells from 50 nmol/L Tf increased linearly with time. The rate of Fe uptake from 50 nmol/L Tf by proliferating cells was significantly increased to approximately 250% the rate by stationary cells. Fe uptake by proliferating and stationary cells from 5 μmol/L Tf increased linearly with time. The rate of Fe uptake from 5 μmol/L Tf by proliferating cells was significantly increased to approximately 250% the rate by stationary cells (Table 2).
cells also was significantly increased to approximately 250% that of stationary cells (Table 3).

The molar ratio of Fe to Tf accumulated by proliferating and stationary cells increased with time. At both Tf concentrations, there were no differences in Fe-to-Tf molar ratio between proliferating and stationary cells; however, the Fe-to-Tf molar ratio was double at 5 μmol/L compared with 50 nmol/L Tf; the molar ratio was approximately 14 and 7, respectively.

**TfR1 and TfR2-α mRNA Expression.** Northern blot analysis of poly (A) + selected RNA from HuH7 cells showed that TfR1 mRNA by proliferating cells was increased 300% compared with that of stationary cells (Fig. 5). In contrast to the TfR1, the expression of TfR2-α mRNA measured by Northern analysis was reduced by 50% to 70% in proliferating cells compared with stationary cells (Fig. 6). Similar results were obtained for TfR2-α mRNA expression using semiquantitative RT-PCR (data not shown). Northern analysis using either a probe that detected the 2 isoforms of TfR2 or one that specifically recognized TfR2-α produced similar results (data not shown).

**TfR1, TfR2-α, and Ferritin Protein Expression.** Western blot analysis revealed that TfR1 and TfR2-α protein levels were increased in proliferating cells compared with stationary cells by approximately 300% (Fig. 7) and 200% (Fig. 8), respectively. Proliferation increased ferritin expression by 300% compared with that of stationary cells (Fig. 9).

<table>
<thead>
<tr>
<th>Table 2. Rate of Iron Uptake by Proliferating and Stationary Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rate of Iron Uptake (n = 3)</strong></td>
</tr>
<tr>
<td><strong>TfR1</strong> (50 nmol/L Tf)</td>
</tr>
<tr>
<td><strong>NTR1</strong> (5 μmol/L Tf)</td>
</tr>
<tr>
<td>Proliferating (pmol Fe/mg protein/min)</td>
</tr>
<tr>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Stationary (pmol Fe/mg protein/min)</td>
</tr>
<tr>
<td>0.10 ± 0.02*</td>
</tr>
<tr>
<td>0.60 ± 0.11*</td>
</tr>
</tbody>
</table>

NOTE. Results are expressed as mean ± SE from 3 separate experiments.
*Significant difference compared with proliferating cells, P < .05.

<table>
<thead>
<tr>
<th>Table 3. Transferrin Uptake by Proliferating and Stationary Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transferrin Uptake (n = 3)</strong></td>
</tr>
<tr>
<td><strong>TfR1</strong> (50 nmol/L Tf)</td>
</tr>
<tr>
<td><strong>NTR1</strong> (5 μmol/L Tf)</td>
</tr>
<tr>
<td>Proliferating (pmol Tf/mg protein/90 min)</td>
</tr>
<tr>
<td>2.75 ± 0.20</td>
</tr>
<tr>
<td>8.35 ± 0.45</td>
</tr>
<tr>
<td>Stationary (pmol Tf/mg protein/90 min)</td>
</tr>
<tr>
<td>1.17 ± 0.11*</td>
</tr>
<tr>
<td>3.61 ± 0.13*</td>
</tr>
</tbody>
</table>

NOTE. Results are expressed as mean ± SE from 3 separate experiments.
*Significant difference compared with proliferating cells, P < .05.
Proliferating Cells Stationary Cells

<table>
<thead>
<tr>
<th></th>
<th>5 μg</th>
<th>10 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. TIR1 gene expression by proliferating and stationary HuH7 TIR1 antisense cells. Poly-A mRNA was isolated from HuH7 TIR1 antisense cell total RNA and analyzed for TIR1 (A) and GAPDH (B) mRNA by Northern analysis. TIR1 gene expression values were normalized for GAPDH mRNA expression (C).

Discussion

In this study, a human HuH7 hepatoma cell line with suppressed TIR1 expression was used to study the uptake of Tf-Fe by the TIR1 and NTIR1 pathways. Previously, we showed that there was a 50% to 60% reduction in the expression of TIR1 receptors with corresponding reduction in the uptake of Tf and Fe by the HuH7 TIR1 antisense cells compared with wild-type cells. Whereas Fe uptake by the NTIR1-mediated process was similar for both HuH7 TIR1 antisense and wild-type cells, indicating that this process is independent of TIR1. Similarly, in this study, we found that irrespective of the state of proliferation of the HuH7 TIR1 antisense cells, there was evidence of TIR1-mediated uptake of Tf-Fe. At low Tf concentration there was an initial binding of Tf and Fe to the cell membrane at a ratio of 1:2, consistent with binding of diferric Tf to its receptor. This was followed by a rapid internalization of Tf into the cell, reaching a steady state after 30 minutes. In contrast, Fe uptake increased in a linear manner with time. Because there was a net accumulation of Fe relative to Tf by the cell, this indicates the Fe was released from Tf to the cytoplasm, that the Tf recycled to the cell surface and was released at the cell membrane, enabling continued uptake of Tf-Fe. These characteristics are consistent with receptor-mediated endocytosis of Tf-Fe by the TIR1 pathway.

During proliferation, the uptake of Tf-Fe by the TIR1 pathway increased compared with stationary cells. It is possible that the increased uptake of Fe could be caused by increased efficiency of TIR1, that is, increased recycling of TIR1 or increased rate of release of Fe from Tf. We assessed this possibility by comparing the $V_{\text{max}}$ for Fe uptake and $B_{\text{max}}$ for Tf binding as an index of TIR1 efficiency and found no difference during proliferation, suggesting other factors such as increased expression of TIR1.

Proliferating Cells Stationary Cells

<table>
<thead>
<tr>
<th></th>
<th>5 μg</th>
<th>10 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIR2-α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. TIR2-α gene expression by proliferating and stationary HuH7 TIR1 antisense cells. Poly-A mRNA was isolated from HuH7 TIR1 antisense cell total RNA and analyzed for TIR2-α (A) and GAPDH (B) mRNA by Northern analysis. TIR2-α gene expression values were normalized for GAPDH expression (C).
are responsible for the increased uptake of Fe and Tf binding with proliferation. Therefore, we measured TfR1 expression at both the mRNA and protein levels and showed that these were equally elevated compared with stationary cells. This supports our findings that the increased expression of TfR1 in proliferating cells was proportional to the increased Fe uptake and Tf binding by the TfR1-mediated process. It is also consistent with other studies on regenerating rat liver cells after partial hepatectomy and hepatocytes during rat liver regeneration, which showed that proliferating hepatocytes express increased number of receptors per cell when compared with stationary cells.

Cell proliferation–dependent transcription of TfR1 expression has been shown and attributed to a 100 base-pair sequence lying upstream from the transcriptional start site. Within this region are Ap1, cyclic adenosine monophosphate–responsive element, Sp1, hypoxia response element, and TfR1 transcriptional control element. The transcription factors that bind these regulatory elements in TfR1 to enhance transcription respond to growth factors found in abundance during proliferation. In addition, ferritin gene expression also was responsive to proliferation. Previous investigators have shown that the induction of ferritin synthesis by Fe is regulated at both transcriptional and translational lev-
It has been shown that ferritin synthesis increases in proliferating lymphocytes, raising the possibility that the promoter regions of ferritin and TfR1 share common elements enabling enhanced transcription during proliferation.36,37

Both TfR1 and ferritin expression are also known to be regulated by posttranscriptional control mechanisms. Posttranscriptional control involves cytosolic Fe regulatory proteins reacting to low cellular Fe levels by binding to Fe-responsive element in the 3' and 5' untranslated region encoding the TfR1 and ferritin mRNAs, respectively. This leads to stabilization of the TfR1 mRNA enhancing translation while blocking translation of ferritin. Collectively, this control mechanism leads to increased uptake of Fe and reduced ferritin to store the Fe. Therefore, under these circumstances there is inverse expression of TfR1 and ferritin protein. However, because this was not observed in the present study, it suggests that the control of these proteins does not occur by posttranscriptional mechanisms.

There is considerable evidence to suggest that a number of different types of cells, including hepatoma cells, hepatocytes, fibroblasts, and melanoma cells, take up Tf-Fe by NTfR1 process at Tf concentration greater than that required to saturate TfR1.39 Evidence for the NTfR1 pathway has been that hepatic cells still take up Tf-Fe when TfR1 expression is inhibited by TfR1 antisense expression vector (Figs. 1, 2, and 4),5,20,40 and by using N-terminal half Tf molecules that do not bind to TfR1.41 Supporting this finding in CHO cells that do not express TfR1, uptake of Tf-Fe was still observed.42 Also in Fe overload disorders such as hereditary hemochromatosis, hepatocytes do not express TfR143,44 but continue to accumulate Fe presumably by an NTfR1 pathway. In the Hfe knockout murine model of hereditary hemochromatosis in contrast to TfR1, TfR2 expression remains high and may be involved in Fe uptake by NTfR1.45

In this study, we showed for the first time that Tf-Fe uptake and Tf binding by the hepatoma cells by the NTfR1-mediated process were both saturable, indicating that this process was mediated by a low-affinity Tf receptor. Uptake of Fe and Tf binding by the NTfR1 process had many characteristics of high-affinity TfR1-mediated process. That is, rapid initial binding of Tf by the cells, reaching a steady state after 30 minutes incubation, whereas Fe uptake increased with time resulting in the net accumulation of Fe by the cell. Given that the binding of Tf and the uptake of Fe by the TfR1-mediated pathway have similar characteristics to that observed for the NTfR1-mediated pathway, it suggests the NTfR1-mediated uptake of Tf-Fe also may involve a receptor-mediated endocytic mechanism possibly involving the low-affinity TfR2-α.

To test this hypothesis, we assessed the effects of proliferation on Tf-Fe uptake by NTfR1 and TfR2 expression. We showed that during proliferation, Tf binding and Fe uptake by the NTfR1 pathway was increased compared with stationary cells. The Fe-to-Tf molar ratio by NTfR1 was similar for both stationary and proliferating cells, suggesting Fe uptake may be elevated by increased expression of a Tf binding protein such as TfR2-α. Indeed, in proliferating cells the TfR2-a protein expression was increased and correlated with increased uptake of Fe by the NTfR1 pathway. These findings support the idea that TfR2-α may be involved in the uptake of Tf-Fe by NTfR1 pathway.

Surprisingly, Northern blot analysis and semi quantitative PCR showed that TfR2-α mRNA expression decreased during proliferation. The low levels of TfR2-α mRNA expression despite high protein expression and increased uptake of Tf-Fe by proliferating cells suggests that the TfR2-α protein may operate more efficiently during proliferation. One possibility is that during proliferation the protein is stabilized and resistant to degradation. Alternatively, the efficiency of translation could be increased during proliferation; however, this is unlikely to involve an Fe regulatory element/Fe regulatory protein mechanism because the TfR2-α mRNA lacks an Fe regulatory element.18 These findings suggest that the control of TfR2-α gene expression differs from TfR1 and ferritin during proliferation.

In another study, proliferation-dependent increase in TfR2-α mRNA levels was observed in osteosarcoma cell line.18 However, in the present study transcription of TfR2-α as assessed by the steady-state levels of TfR2-α mRNA was reduced by proliferation in a hepatocyte-derived cell line. A possible explanation for this difference may be found in a recent study showing that the amount of the normally highly expressed hepatic transcription factor CCAAT enhancer-binding protein (C/EBP-α) is reduced during proliferation.46 Interestingly, reporter assays revealed that C/EBP-α enhances TfR2-α promoter activity, and so it would be predicted that proliferation would reduce TfR2-α mRNA expression in hepatocytes. To our knowledge, it is unknown whether TfR1 contains elements for C/EBP-α binding; if not, this may account for the differential expression of TfR1 and TfR2-α during proliferation.

Recently, patients with mutations in TfR2 (hemochromatosis type 3) have been identified that have elevated Tf saturation and liver Fe overload.47 TfR2 mutations would be predicted to lead to loss or reduction in function; however, this has not been observed in these patients because
the liver still loads with Fe, suggesting a role for TfR2 in the regulation of Fe metabolism. How TfR2 regulates Fe metabolism is unclear. One possibility is that TfR2 expression or TfR2-mediated uptake of Tf-Fe by the liver may regulate the synthesis of the newly identified hepatic peptide hepcidin, which may act as a signaling molecule in regulation of liver Fe storage levels and Fe absorption.

In summary, this study has revealed an increase in Tf-Fe uptake by TfR1, and TfR1 pathways were associated with increased TfR1 mRNA and protein and increased TfR2 protein by proliferating HuH7 hepatoma cells. The reciprocal relationship between the expression of TfR2 mRNA and protein by the hepatoma cells in proliferative and stationary states suggests a different mechanism of gene regulation to TfR1. These findings suggest that TfR2 mediates an increase in Tf-Fe uptake by the NTfR1 process during proliferation.

Acknowledgment: The authors thank Dr. H. Kawabata for the generous donation of the TfR2-α cDNA template and antibody.

References

30. Owens D, Kuhn LC. Noncoding 3' sequences of the transferrin receptor gene are required for mRNA regulation by iron. EMBO J 1987;6:1287-1293.


