Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells

Hiroshi Kawabata, Tsuyoshi Nakamaki, Pranvera Ikonomi, Reginald D. Smith, Rasha S. Germain, and H. Phillip Koeffler

Iron is essential for cell proliferation, heme synthesis, and a variety of cellular metabolic processes. In most cells, transferrin receptor–mediated endocytosis is a major pathway for cellular iron uptake. Recently, transferrin receptor 2 (TfR2), another receptor for transferrin, was cloned. High levels of expression of TfR2 messenger RNA (mRNA) occur in the liver, as well as in HepG2 (a hepatoma cell line) and K562 (an erythroid leukemia cell line). In this study, TfR2 mRNA expression was analyzed in hematological cell lines, normal erythroid cells at various stages of differentiation, and leukemia and preleukemia cells. High levels of TfR2 expression occurred in all of the erythroid cell lines that were examined. Erythroid-specific expression of TfR2 protein in bone marrow cells was confirmed by immunohistochemical staining. Expression of TfR2 mRNA was high in normal CD34+ erythroid precursor cells, and levels decreased during erythroid differentiation in vitro. Levels of expression of TfR2-α mRNA were significantly higher in erythroleukemia (M6) marrow samples than in nonmalignant control marrow samples. In addition, relatively higher levels of TfR2-α mRNA expression occurred in some samples of myelodysplastic syndrome that had erythroid hyperplasia in bone marrow, acute myelogenous leukemia M1, M2, and chronic myelogenous leukemia. Expression profiles of normal members of the erythroid lineage suggest that TfR2-α may be a useful marker of early erythroid precursor cells. The clinical significance of TfR2-α expression in leukemia cells remains to be determined. (Blood. 2001;98:2714-2719)

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Materials and methods

Cell lines
TfR1-deficient CHO-TRVb cells were kindly provided by Dr T. McGraw. MOLT-16 (T-lymphoid), 3 NB4 (a myeloid cell line derived from a patient with acute myelogenous leukemia [AML]–M3), 10 and KCL22 (a myeloid cell line from a patient with chronic myelogenous leukemia [CML]) were kindly gifts from Drs J. Minowada, M. Lanotte, and I. Miyoshi, respectively. Erythroid cell lines HEL-R (from a patient with erythroleukemia), 12

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DCI-M1 (from a patient with erythroleukemia),12 and KU-812-F (derived from a patient with CML and spontaneously differentiating toward erythroid lineage in vitro)13,14 were kindly provided by Dr T. Papayannopoulou. KG-1 (a myeloid cell line) was established by our group.15 TfR2 stable transfectants of CHO-TRb cells were established as previously described.4 Raji (B-lymphoid), U937 (monoblastoid), HL-60 (myeloid), and K562 (erythromegakaryocytoid) cell lines were obtained from American Type Culture Collection (Manassas, VA).

Immunohistochemistry

Cytospin specimens of CHO-TRb cells (stably transfected with either TfR1, TfR2, or neomycin-resistant control plasmid) and normal bone marrow (BM) mononuclear cells were fixed with acetone and then incubated with either diluted rabbit anti-TfR2 antiserum or normal rabbit serum for 30 minutes, followed by immunohistochemical staining with Envision/AP (Dako, Carpinteria, CA). The cells were counterstained with Mayer hematoxylin (Wako Pure Chemicals, Osaka, Japan).

Preparation of human erythroid cells at various stages of differentiation

Human erythroid cells from buffy coats were cultured as previously described.16 Briefly, the mononuclear cells were obtained by density centrifugation in lymphocyte separation media (Organon Teknika, Durham, NC). The cells were cultured in the Phase I α-Eagle minimum essential medium (α-MEM) (Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), 10% conditioned media prepared from supernatant of 5637 carcinoma bladder cells, and 1 μg/mL cyclosporin A (Sigma). After 7 days' incubation, the cells were washed with phosphate-buffered saline and transferred to Phase II α-MEM media containing 30% FBS; 10% bovine serum albumin; 10−5 M 2-mercaptoethanol; 10−6 M dexamethasone; 0.033 g/L (33 μg/mL) holotransferrin; 10 ng/mL stem cell factor (Sigma); and 1000 U/L (1 U/mL) erythropoietin (Amgen, Thousand Oaks, CA). After 4 days in Phase II culture, the cells were collected and incubated for 30 minutes with a cocktail of antibodies against the following cell surface markers: CD2, CD19, CD33, and CD66b. After incubation for an additional 30 minutes with Stemsep Magnetic colloid, the cells of interest were isolated by negative selection with a 0.6 Tesla magnet (Stem Cell Technologies, Vancouver, BC, Canada). Stage-specific cell populations (CD34+/A4, and glycoporphin A (GPA)/A4) have been isolated from the erythroid population by positive selection with anti-CD34 and anti-GPA antibodies (Stem Cell Technologies), respectively. To obtain erythroid cells from a later stage of maturation, both cell depletion and positive selection by means of GPA have been applied to erythroid cultures that have been incubated for 10 days in Phase II (GPA)10. Alternatively, the CD34+ A4 progenitors were cultured in the same media for up to 14 days. The purity and homogeneity of these populations were confirmed by flow-cytometry analysis.

Quantitative RT-PCR for human erythroid cells at various stages of differentiation

The mRNA was extracted from human erythroid cells prepared as above, and complementary DNA (cDNA) was synthesized. Real-time quantitative PCR (Q-PCR) was performed with primers specific for TfR1 (5′-AAA ATCCGGTGAGGCACAG-3′) and 5′-CTCTTAAATGCGGGAGC-3′), TfR2 (5′-TACACACGTTCGACACAA-3′ and 5′-AGTAACCCACTTGAGGTC-3′), and TfR2-α (5′-ACCTTGAGGAGGAAAGGAA-3′ and 5′-CGACGAGCTGAGGAGGAGG). Primer specificity was confirmed by restriction endonuclease and agarose gel analysis. Sybr green I dye (Molecular Probes, Eugene, OR) was used as the reporter dye for Q-PCR, which was performed in a PE Biosystems SDS 7700 thermal cycler (PerkinElmer, Boston, MA). Results are presented as attomoles per microgram mRNA.

RT-PCR for clinical samples

From a collection at Showa University School of Medicine (Tokyo, Japan), 107 leukemia and preleukemia samples from 90 patients were analyzed for TfR1, TfR2-α, and TfR2-β expression by semiquantitative RT-PCR. Either BM or peripheral blood mononuclear cells (PBMCs) were used. These samples were from patients with AML (38 BMs and 25 PBMCs); CML (chronic or accelerated phase, 5 BMs and 4 PBMCs); acute lymphocytic leukemia (ALL) (3 BMs and 5 PBMCs); myelodysplastic syndromes (MDSs) (20 BMs and 6 PBMCs); and aplastic anemia (1 BM). The French-American-British classification of AML and MDS is presented here, along with an enumeration of the numbers and types of samples we obtained for each: M1, undifferentiated (4 BMs and 3 PBMCs); M2, myeloblastic (6 BMs and 3 PBMCs); M3, promyelocytic (6 BMs); M4, myelomonocytic (6 BMs and 6 PBMCs); M5a, poorly differentiated monoblastoid (3 BMs and 1 PBMC); M5b, well-differentiated monoblastic (4 BMs and 5 PBMCs); M6, erythroleukemia (7 BMs); refractory anemia (RA) (3 BMs); RA with ring sideroblasts (RARS) (4 BMs); RA with excess blasts (RAEB) (8 BMs and 4 PBMCs); RAEB in transformation (RAEBT) (5 BMs and 2 PBMCs). As controls, 8 normal BM (idiopathic thrombocytopenic purpura, iron deficiency anemia, and normal BM) and 6 normal PBMC samples were also analyzed. The semiquantitative RT-PCR was performed essentially as previously described.17 Primers and cycle numbers were as follows: for TfR1, primers 5′-AGGAACCGAAGTTGTTCTCAGGTGA-3′ and 5′-ATCACTATGATCCCGAGT-3′, 22 cycles; for TfR2-α, primers 5′-CTGGTTAGAGGAACTGTTA-3′ and 5′-CCACACCGTGTCGAGGTTG-3′, 22 cycles; for TfR2-β, primers 5′-AGCTCTGGCTACCTTCC-3′ and 5′-TGTAAGGGACATGGGCT-3′, 25 cycles; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primers 5′-TACATGGCCTTTTCTTTGTT-3′ and 5′-AAGAGAGGACCTCTCCACC-3′, 13 cycles. The PCR products were transferred to nylon membranes after agarose gel electrophoresis and hybridized with 32P-labeled cDNAs, and the ratio of band intensities of TfR1, TfR2-α, and TfR2-β versus GAPDH were calculated with the use of a densitometer.

Other methods

Northern blot analysis was performed by means of TfR1, TfR2, and GAPDH cDNA probes and standard protocols.4,11 Statistical analysis was performed by means of unpaired Student t test.

Results

Expression of TfR2 mRNA in hematopoietic cell lines

Our previous study showed that high levels of TfR2 expression occurred in K562, an erythroid leukemia cell line derived from a patient with CML, whereas expression of TfR2 mRNA was not detectable in the myeloid cell lines KG-1, U937, and HL-60 by a standard Northern analysis.4 To examine whether TfR2 expression is specific for erythroid cells in the hematopoietic system, we performed Northern analysis using a variety of hematopoietic cell lines, including 4 erythroid cell lines: HEL-R, KU-812-F, OCI-M1, and K562. Among the cell lines that we tested, all of the erythroid cell lines expressed high levels of TfR2 mRNA, while all the lymphoid (Raji and MOLT-16) and myeloid (U937, NB4, HL-60, KCL22, and KG-1) cell lines expressed either low or undetectable levels of TfR2 mRNA (Figure 1). In contrast, TfR1 expression was detectable in all the cell lines that we tested, though levels of TfR1 mRNA expression were very high in OCI-M1 cells and were relatively low in Raji, U937, HEL-R, and K562 cells (Figure 1).

Immunohistochemical staining of TfR2-transfected CHO-TRb and normal BM cells

To examine if expression of TfR2 is lineage specific among hematopoietic cells, we used the immunohistochemical technique. First we tested our anti-TIR2 antibody for this technique using CHO-TRb cells that had been stably transfected with either TfR1 or TfR2-α. Cytospin
cally increased during erythrocytic maturation between GPA
d10 and GPA
d4, while Tfr2 mRNA gradually decreased during erythrocytic differentiation. The primers that we used for
analysis, indicating that these cells were at either the erythroid
colonies or the erythroid stage. The results from
Q-PCR are shown in Figure 3A. Levels of Tfr1 mRNA dramati-
cally increased during erythrocytic maturation between GPA
d4 and GPA
d10, while Tfr2 mRNA gradually decreased during erythrocytic differentiation. The primers that we used for Tfr2 could
amplify both $\alpha$ and $\beta$ forms, although our previous study indicated
that the majority of Tfr2 transcripts in erythroid cell lines were the
$\alpha$ form. To confirm the expression profile of the $\alpha$ transcripts
during normal erythrocytic differentiation, we designed another set of
primers that can amplify only the $\alpha$ form. For this analysis,
CD34+/d4 cells were cultured in the presence of erythropoietin,
and the cells were harvested at days 4, 6, 7, 10, 14, and 18. The cells
harvested at day 6 expressed CD36 and GPA but not CD34, so this
population was the equivalent of the population of GPA
d4 in Figure 3A. The cells harvested at day 10 expressed both GPA and
CD71, and these cells were the equivalent of the GPA
d10 cells in Figure 3A. Results of quantitative RT-PCR shown in Figure 3B
demonstrated that, in agreement with our first analysis, the levels of
Tfr2-$\alpha$ transcript declined as the erythroid progenitors matured.

Expression of Tfr2 mRNA in BM and PBMCs from patients
with hematological disorders

We analyzed 107 samples from 90 individuals with leukemia and
preleukemia together with 8 nonmalignant BM and 6 normal
PBMC samples for expression of Tfr1, Tfr2-$\alpha$, and Tfr2-$\beta$
mRNAs. Some of the samples were taken serially from the same
patients during disease progression. Expression levels of Tfr2-$\alpha$
were higher in nonmalignant BM samples than in normal PBMC
samples ($P = .038$; Figure 4A, shaded bars). Among the BM
samples, levels of Tfr2-$\alpha$ expression in M6 were clearly higher

![Figure 1. Northern blot analysis of Tfr expression in hematopoietic cell lines.](image)

![Figure 2. Immunohistochemical staining for Tfr2.](image)
than those of nonmalignant BM samples ($P = .247$; Figure 4A).

High levels of expression of $TfR2$-a (greater than 60% of K562) occurred in 13 samples: 1 M2-BM, 4 M6-BM (erythroleukemias), 1 CML-BM, 2 RARS-BM, 2 RAEB-BM, 1 RAEBT-BM, 1 M1-PB, and 1 CML-PB.

Differential cell counts of the BM were available from 9 of the 11 BM samples that showed high levels of $TfR2$-a expression (Table 1; all the M6 cases are also shown). Samples 28 and 198 were obtained from the same patient at different times: when initial diagnosis was made (sample 28) and 4 months later when blast cells became nearly 70% in the BM (sample 198). Similarly, samples M6-3 and M6-4 were from another patient at different times: at the initial diagnosis (M6-3) and at relapse (M6-4). The M6-6 sample was obtained after the individual had received repeated units of red blood cell transfusion, which may have suppressed the marrow erythroid number. Most cases with high levels of expression of $TfR2$-a mRNA showed BM erythroid hyperplasia, except for sample 219, a CML case that had myeloid hyperplasia. In samples 28 and 198 (the same M6 patient), $TfR2$-a expression decreased (from 141% to 3% of K562) during disease progression (myeloblasts increased and erythroid cells decreased during this period). Among PB samples, one M1 and one CML (accelerated phase) sample showed high levels of $TfR2$-a expression; the M1 sample contained 98% blast cells, and the CML sample contained immature myeloid cells that included blast cells.

The profile of $TfR2$-b expression largely paralleled levels of the $a$ form, being significantly lower in AML (except M5a and M6) and ALL as compared with nonmalignant BM samples (Figure 4B).

Unlike $TfR2$-a, expression of the $\beta$-form was not increased in M6 samples. Also, the expression profile of $TfR1$ mRNA was largely, but not always, similar to that of $TfR2$-a (Figure 4C). For example, in sample M6-6 (Table 1), expression of $TfR1$ was very elevated (158% of K562), and levels $TfR2$-a were low (only 9% of K562).

**Discussion**

Among the hematological cell lines that we tested, high levels of $TfR2$ occurred only in erythroid cell lines (Figure 1).
Table 1. Bone marrow cell classification of erythroleukemia (M6) cases as well as cases with high level of expression of TfR2-α messenger RNA

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Diagnosis</th>
<th>Expression (% K562)</th>
<th>BM (%)</th>
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<tr>
<td></td>
<td></td>
<td>TfR2-α</td>
<td>TfR2-β</td>
</tr>
<tr>
<td>28*</td>
<td>M6</td>
<td>141</td>
<td>311</td>
</tr>
<tr>
<td>198</td>
<td>M6</td>
<td>3</td>
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<td>M6</td>
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<td>103</td>
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<td>279</td>
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<td>63</td>
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<td>965</td>
</tr>
<tr>
<td>55*</td>
<td>RAEBT</td>
<td>61</td>
<td>148</td>
</tr>
</tbody>
</table>

Nonmalignant

BM, mean ± SD

Expression of TfR1, TfR2-α, and TfR2-β messenger RNA was analyzed by semiquantitative reverse-transcriptase polymerase chain reaction and shown as a percentage of the level detected in K562 cells. As reference, the mean ± SD of expression levels of nonmalignant marrow samples are shown in the bottom row (control).

*Samples that had high levels of expression of TfR2 mRNA (greater than 60% K562).

that expression of this gene may be selective to the erythroid lineage of hematopoietic cells. This idea was supported by our immunohistochemical staining of normal BM cells using anti-TfR2 antibody, in which a majority of erythroblasts, but not myeloid cells, were clearly stained. These results are consistent with our previous study that showed enhancement of murine TfR2 promoter activity by GATA-1. GATA-1 is highly expressed in erythroid cells as well as in megakaryocytes, eosinophils, and mast cells, and the putative GATA-1–binding sites of TfR2 are well conserved between human and mouse. We observed a few large cells, probably megakaryocytes, that were positive for TfR2 staining (Figure 2G).

In the leukemic and preleukemic BM samples, levels of TfR2 mRNA roughly correlated with the proportion of erythroid cells in the marrow. High levels of TfR2-α mRNA expression occurred in 4 of 7 M6 erythroleukemias, 1 of 6 M2, 1 of 5 CML, 2 of 4 RAES, 2 of 8 RAEB, and 1 of 5 RAEBT samples. Among them, marrow cell classification was available in 9 cases; all but 1 CML case, had an increased percentage of erythroid cells in the BM (Table 1) (erythroid cells greater than 30%), which is consistent with our hypothesis that the levels of TfR2 expression may be related to mass of immature erythroid cells. But this was not always the case. Two CML cases with myeloid hyperplasia (one BM and one PBMC) and one individual with AML-M1 (PBMCNs with 98% blast cells) also highly expressed TfR2-α mRNA (Table 1 and data not shown). These results suggest that, in addition to immature erythroid cells, some myeloid, nonerythroid leukemia cells also expressed high levels of TfR2-α. On the other hand, sample M6-5 had low levels of TfR2 mRNA but had remarkable erythroblast hyperplasia (greater than 80% erythroid cells) (Table 1).

In MEL cells, we have shown that expression of TfR2 decreased and TfR1 increased during dimethyl sulfoxide–induced erythroid differentiation. Similar expression profiles were observed in normal human erythroid cells as they differentiated in vitro in the presence of erythropoietin (Figure 3). The CD34+ erythroid precursors expressed high levels of TfR2 and very low levels of TfR1 mRNAs. During their differentiation, levels of TfR2 mRNA decreased gradually and expression of TfR1 mRNA increased dramatically (Figure 3B). TfR2 has at least 2 transcripts, α and β, and our previous observations indicated that the majority of TfR2 transcripts were the α form. The expression profile of TfR2-α during erythroid differentiation was almost the same as that of TfR2 (Figure 3B).

We have shown that TfR2-α, similarly to TfR1, can mediate cellular iron uptake and support cell growth in TIR-deficient CHO cells. CHO cells stably expressing TfR2-α were grown in nude mice; the resulting tumors developed much faster and became much larger than those of neomycin-resistant control cells. This reflects the growth-supporting effects of TfR2-α. However, the physiological function of TfR2-α is still unclear. Prior studies have demonstrated that expression of TfR1 was downregulated by iron loading and upregulated by iron deficiency in the liver and K562 cells. The main mechanism of this regulation is through binding of iron-regulatory proteins (IRPs) to the iron-responsive elements (IREs) of the 3′-untranslated region of the TfR1 mRNA. In a low-iron environment, IRPs bind to the IREs of the TfR1 mRNA and stabilize it, whereas in the presence of excess iron IRPs are released from IREs, resulting in degradation of these transcripts.

In contrast, TfR2 is not known to be regulated by cellular iron status, has no IREs in the region of the gene, and is expressed constitutively in the liver and K562 cells. These TfR2 expression profiles raise the question of whether the only function of TfR2-α is to facilitate intracellular iron uptake.

Recently, Camaschella et al reported on patients with hereditary hemochromatosis from 4 families, who had homozygous nonsense mutations (Tyr250Xaa, Glu60Xaa, or Met172Lys) of the TfR2 gene. Both Tyr250Xaa and Met172Lys mutations affect both the α and β forms of TfR2, and the Glu60Xaa mutation affects only the α form. These reports are paradoxical in terms of our previous studies, which suggested that a function of TfR2 was to enhance cellular uptake of iron; thus, the authors proposed that the major function of TfR2 may be involved in iron regulation rather than iron uptake. Intriguingly, according to the report by Camaschella et al, none of the hemochromatosis patients with homozygous TfR2 mutations showed erythroid abnormalities, although our current study showed high levels of expression of TfR2 in erythroid precursors. TfR1 or other molecules may be able to substitute for the function of TfR2 in erythroid precursors of these patients.

From the current study, we believe that TfR2 may be a useful marker for early erythroid cells. We also identified several myeloid, nonerythroid leukemia cases in which levels of expression of TfR2 were relatively high. Expression of TfR2 may have some relevance to clinical features of these cases. Functional significance of the TfR2 gene in hematopoietic cells remains to be delineated by study of TfR2-deletional mice.

Acknowledgments

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References

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