Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells

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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 TfR2α mRNA were significantly higher in erythroleukemia (M6) marrow samples than in nonmalignant control marrow samples. In addition, relatively higher levels of TfR2α 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 TfR2α may be a useful marker of early erythroid precursor cells. The clinical significance of TfR2α expression in leukemia cells remains to be determined. (Blood. 2001;98:2714-2719)

Introduction

Iron is essential for a variety of physiological activities of cells, such as electron transport and DNA synthesis, and it is used as a cofactor of cytochromes, aconitases, ribonucleotide reductase, and heme proteins.1,2 Transferrin receptor 1 (TfR1) is a type II membrane protein that mediates cellular iron uptake. In the serum, most iron exists in a transferrin-bound form.3 On the cell surface, iron-bound transferrin binds to TfR1; this is followed by internalization of this complex.

Recently, another receptor for transferrin, transferrin receptor 2 (TfR2), was cloned in our laboratory.4 At least 2 alternatively spliced forms of transcripts, α and β, are transcribed from the TfR2 gene. TfR2-α protein is a type II membrane–integrated glycoprotein similar to TfR1 that can mediate iron uptake through transferrin. TfR2-β lacks both intracellular and transmembrane domains and may be an intracellular protein. Although TfR2-β transcripts were detectable by a very sensitive reverse-transcriptase polymerase chain reaction (RT-PCR), it was not detectable by Northern blot analysis. In contrast, TfR2-α was easily detectable by Northern blotting in the liver and in K562 cells, indicating that the α-form transcript was far more prevalent than the β-form in these cells. Among various tissues and cell lines that we tested, high levels of expression of TfR2 messenger RNA (mRNA) occurred only in the liver, and in HepG2 hepatoma and K562 erythroid cell lines, suggesting that its expression was tissue specific.4,5

Considering our limited knowledge of the expression profile of TfR2, we asked the following questions: (1) Is TfR2 expression, as a previous report6 suggested, specific to erythroid cells within the hematopoietic population? (2) Iron is essential for cell growth, and we previously showed that expression of TfR2-α supported cell growth in TfR-deficient Chinese hamster ovary (CHO) cells.7 Therefore, is expression of TfR2-α particularly predominant in hematological diseases such as leukemia? (3) In murine erythroleukemia (MEL) cells, TfR1 was upregulated and TfR2 was downregulated as the cells underwent erythroid differentiation.6 Do these changes occur during normal erythroid differentiation? In this study, we address these questions by analyzing TfR2 expression in normal erythroid precursors as well as in preleukemia and leukemia cells.

Materials and methods

Cell lines

TfR1-deficient CHO-TRVb cells were kindly provided by Dr T. McGraw.6 MOLT-16 (T-lymphoid),8 NB4 (a myeloid cell line derived from a patient with acute myelogenous leukemia [AML]–M3),9 and KCL22 (a myeloid cell line from a patient with chronic myelogenous leukemia [CML])10 were kind 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. Papayannopoulos. KG-1 (a myeloid cell line) was established by our group.15 TfR2 stable transfectants of CHO-TRVb cells were established as previously described.4 Raji (B-lymphoid), U937 (monoblastoid), HL-60 (myeloid), and K562 (erythroleukagaryocytic) cell lines were obtained from American Type Culture Collection (Manassas, VA).

**Immunohistochemistry**

Cytospin specimens of CHO-TRVb 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 antiserum7 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; 10⁻² 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. For incubation for an additional 30 minutes with Stemsep Magnetic colloid, the cells of interest were isolated by negative selection with a 0.6 μL Tesla magnet (StemCell Technologies, Vancouver, BC, Canada). Stage-specific cell populations (CD34⁺/dd, and glycophorin A (GPA/dd) 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/dd). Alternatively, the CD34⁺/dd 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 ATCCGGGTAGGCACACG-3’ and 5’-CCCTTTAATGCAGGAGGAC AA-3’); *TfR2* (5’-TACCCATCTCCCTGACCA AA-3’ and 5’-ATGACAC CCACTGGAGG TC-3’); and *TfR2*-α (5’-ATCCGTGGAAGGAAAGG GAA-3’ and 5’-CGACGTAGCCGATGAGGAG-3’). 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 monoblastic (3 BMs and 1 PBMC); M5b, well-differentiated monocytic (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 nonmalignant 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’-AGGACCGA GTCTCCATTGGA-3’ and 5’-ATCACACTGGACTCCCCGATG-3’, 22 cycles; for *TfR2*-α, primers 5’-GTTGGTTAGGGATTGTCCA A-3’ and 5’-CCACACGTGTCGATCCTGTCGAGG-3’, 22 cycles; for *TfR2*-β, primers 5’-ACGCTTCTGGATCCTCCT-3’ and 5’-TGG TGGGACGTAGACGTCA-3’, 25 cycles; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primers 5’-ATAAGGCAATGGGACTCCC AAGTCA-3’ and 5’-AAGGAGGATTCTTCCCCCT-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.4,18 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 erythroleukemia 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-TRVb 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-TfR2 antibody for this technique using CHO-TRVb cells that had been stably transfected with either *TfR1* or *TfR2*-α. Cytospin...
cally increased during erythrocytic maturation between GPA
day 1 and day 4 cells were cultured in the presence of erythropoietin,
and colonies were counted. GPA
day 10 cells 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 both GPA and CD71, and these cells were the equivalent of the population of GPA+/d4 cells in Figure 3A. The cells harvested at day 10 expressed both GPA and CD71, and these cells were the equivalent of the population of 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 TIR2-α transcript declined as the erythroid progenitors matured.

Expression of TIR2 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 TIR1, TIR2-α, and TIR2-β mRNAs. Some of the samples were taken serially from the same patients during disease progression. Expression levels of TIR2-α were higher in nonmalignant BM samples than in normal PBMC samples (P = .038; Figure 4A, shaded bars). Among the BM samples, levels of TIR2-α expression in M6 were clearly higher
than those of nonmalignant BM samples \( (P = .247; \text{Figure 4A}). \) High levels of expression of \( Tfr2-\alpha \) (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 \( Tfr2-\alpha \) 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 \( Tfr2-\alpha \) 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), \( Tfr2-\alpha \) 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 \( Tfr2-\alpha \) expression; the M1 sample contained 98% blast cells, and the CML sample contained immature myeloid cells that included blast cells.

The profile of \( Tfr2-\beta \) expression largely paralleled levels of the \( \alpha \) form, being significantly lower in AML (except M5a and M6) and ALL as compared with nonmalignant BM samples (Figure 4B). Unlike \( Tfr2-\alpha \), 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 \( Tfr2-\alpha \) (Figure 4C). For example, in sample M6-6 (Table 1), expression of \( Tfr1 \) was very elevated (158% of K562), and levels \( Tfr2-\alpha \) 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). This suggests...
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 studies, which suggested that a 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 TfR2 mRNA expression 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, none erythroid leukemias 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.

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