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Reticulocyte transferrin receptor (TfR) expression and contribution to soluble TfR levels

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Background and Objectives. Transferrin receptor (TfR) expression in erythroid cells is regulated by a number of factors, including iron status and erythropoietin (Epo) stimulation. However, the impact of these factors on reticulocyte TfR expression *in vivo* has never been studied. A soluble form of TfR (sTfR) is present in serum in proportion to the mass of cellular TfR. Although sTfR shedding by reticulocytes and erythroblasts has been demonstrated *in vitro*, the contribution of reticulocyte TfR to serum sTfR has never been evaluated *in vivo*.

Design and methods. We measured directly the total number of reticulocyte TfR in normal rats of different age and iron status, as well as in animals experiencing various conditions and treatments aimed at altering erythropoietic activity and iron status, including rHuEpo therapy, hemolytic anemia, phlebotomies, hypertransfusions, thiamphenicol-induced red cell aplasia or inflammation. In addition, we examined the impact of repeated hypertransfusions with normal, reticulocyte-poor and reticulocyte-rich blood on serum sTfR levels.

Results. The number of TfR molecules per reticulocyte was around 50,000 in young rats but was around 100,000 in older animals. These values remained constant in most conditions and in particular were not influenced by iron supplementation or iron overload. However, functional iron deficiency as well as rHuEpo therapy resulted in increased reticulocyte TfR expression. In addition, TfR numbers in reticulocytes were elevated in the early phase of recovery after acute hemolysis or red cell aplasia but normalized soon after. Hypertransfusion experiments clearly demonstrated that reticulocytes can contribute substantially to sTfR levels *in vivo*.

Interpretation and conclusions. TfR numbers are regulated *in vivo* by the same factors as *in vitro*, in particular iron deficiency and erythropoietin stimulation. Circulating reticulocytes contribute significantly to serum sTfR levels.

Key words: transferrin receptor, soluble transferrin receptor, erythropoiesis, iron status, reticulocyte

The transferrin receptor (TfR) mediates uptake of plasma transferrin iron by erythroid as well as all other cell types.[1-3] The functional receptor is composed of two monomers linked by two disulfide bridges to form a molecule of 190,000 Daltons. Virtually all cells, except mature red cells, have TfR on their surface, but the largest numbers are found on the erythron. Many more TfR are seen on CFU-E than on BFU-E[4-6] and their number increases to about 300,000 on early normoblasts and up to 800,000 on intermediate normoblasts,[7] before declining to about 100,000 on reticulocytes and none on mature red cells.[7,8] TfR expression is co-ordinated by a number of factors. Intracellular iron regulates the stability of TfR mRNA by means of an interaction between the iron regulatory proteins (IRP) that sense changes in the chelatable iron pool, and iron-responsive elements (IRE) on TfR mRNA.[2,9] When intracellular iron is abundant, IRP do not bind to IRE, resulting in increased mRNA degradation. When iron is scarce, binding of IRP to IRE stabilizes TfR mRNA. Nitric oxide also modulates IRP activity and thus enhances TfR expression, although this may occur indirectly through changes in intracellular iron.[1,9,10] Erythropoietin (Epo) increases TfR mRNA levels and cell surface expression in erythroid cells through either changes in transcription rates[1] or enhanced binding of IRP-1 to IRE.[11] Hypoxia by itself increases TfR expression independently of IRP activity through a hypoxia-responsive element in the TfR gene.[12,13] However, the impact of iron status and Epo stimulation on reticulocyte TfR expression *in vivo* has never been studied.

A soluble form of the TfR (sTfR) has been identified in animal[14] and human[15] serum. Soluble TfR is a truncated monomer of tissue receptor, lacking its first 100 amino acids, which circulates in the form of a complex of transferrin and its receptor.[14-16] The most important determinant of sTfR levels is marrow erythropoietic activity, which determines variations of as much as 8 times below and 20 times above average normal values in situations characterized by diminished or increased erythropoiesis, respectively.[14,15] Iron status also influences sTfR levels,

which are considerably elevated in iron deficiency anemia[17] and already so in functional iron deficiency, a situation defined by tissue iron deficiency despite adequate iron stores.[18] Soluble TfR has thus been established as a quantitative marker of the mass of cellular TfR. However, the cell population predominantly involved in TfR shedding has not been clearly identified. *In vitro*, truncated sTfR have been shown to be released by tumor cells,[19,20] reticulocytes[20,21] and erythroblasts.[22] The relative contributions of reticulocytes and erythroblasts to sTfR levels have not been determined. These are difficult to clarify because the two categories of cells always change in parallel, except in situations characterized by ineffective erythropoiesis, such as thalassemia major. The fact that the highest sTfR levels have been found in thalassemic patients, in whom reticulocyte counts are quite low, would suggest that the relative contribution of normoblasts to sTfR levels predominates.[15]

We, therefore, carried out a study in rats to examine the effect of iron status and erythropoiesis on the expression of TfR in reticulocytes and to investigate the contribution of reticulocyte TfR to sTfR levels *in vivo*.

Design and Methods

Animals

Male Wistar rats were obtained from Janssen (Beerse, Belgium) and raised at the University of Liège from the age of 3 weeks through to the end of the experiment. All experiments were carried out under ether anesthesia. Animals were fed standard granular food without iron supplements. Hemolytic anemia was produced by intraperitoneal injection of 50 mg acetylphenylhydrazine per Kg body weight, some animals receiving a single dose on day 0 and others 3 doses given on days 0, 4 and 8 to obtain an on-going hemolysis. Red cell aplasia was provoked by intraperitoneal injections of thiamphenicol in one of 2 different schedules: 50 mg b.i.d. for 4 consecutive days or 100 mg once daily for 10 consecutive days. Inflammation was generated by injecting 0.25 mL turpentine oil in each thigh. Iron-deficient erythropoiesis was induced by bleeding animals 3-4 mL on 2 consecutive days as well as on alternate days thereafter to maintain a hematocrit of about 25-30%. In order to suppress erythropoiesis, rats were transfused with 3-4 mL washed packed red cells every other day to maintain a hematocrit of 60-70%. Three types of donor animals were used. Normal unmanipulated donors had reticulocyte counts around 7%. Other donors were previously treated with thiamphenicol to induce red cell aplasia and so procure blood with <2% reticulocytes. A third group of donors received phenylhydrazine twice within an interval of 4 days and blood was collected at the time of peak reticulocytosis (>30%).

Recombinant human erythropoietin (rHuEpo, Recormon®) was kindly provided by Boehringer-Mannheim (Brussels, Belgium). Rats were injected daily with intravenous rHuEpo at a dose of 10, 50 or 150 U/day for up to 20 days. Control animals (0 U rHuEpo) received the same volume of normal saline. Some groups of rats treated with rHuEpo were fed standard food in powder form supplemented with 1% (w/w) carbonyl iron (Sigma Chemical Co., St. Louis, USA) 6 days per week and normal granular food 1 day per week, for a total of 2 weeks (*iron supplemented rats*) or 3 months (*iron overloaded rats*) before as well as during rHuEpo treatment.

Experimental procedures

Blood samples were drawn from a tail vein 3 times per week until the final day of the experiment when ferrokinetic studies were performed. Labeling with ^{59}Fe was accomplished by adding 1 µCi ^{59}Fe citrate to 0.5 mL donor plasma under continuous agitation. The plasma was then incubated for 30 minutes at 37°C before injection into a tail vein. Blood samples were obtained from the tail vein on the opposite side and the rate of radioiron disappearance from circulation ($t_{1/2}$) was determined by least squares regression analysis. From the Hct, serum iron, transferrin saturation and radioiron clearance, the erythron transferrin uptake (ETU, µmol/L whole blood/day) was then calculated.[23,24]

Three hours (6 for hypertranfused animals) after radioiron injection, rats were exsanguinated from the abdominal aorta and perfused with 20-30 mL saline. The spleen, liver and 2 femora were removed and weighed. Whole blood and plasma activities, as well as activity of the liver, spleen and femora were counted. Total plasma and total red cell activities were calculated from the hematocrit, assuming a blood volume of 0.07 mL/g body weight. Erythroid marrow activity was estimated by multiplying the counts of the 2 femora by 6.5.[25] The 2 extremities of the femora were cut and ground and the supernatant was obtained after centrifugation, while the bone marrow was flushed out of the femoral shafts. The two marrow components were combined, counted and the efficiency of marrow extraction was calculated. All calculations accounted for the efficiency of marrow extraction determined for each individual animal.

The whole spleen and extracted bone marrow, weighed aliquots of the liver, as well as washed red cells from 1 mL whole blood were then homogenized and transferrin receptors solubilized at 4°C in a Polytron PT-K homogenizer (Kinematica, Littau, Switzerland) for 1 min at 15,000 rpm and then 0.5-2 min at 24,000 rpm. All samples were homogenized in adequate amounts of 10 mM phosphate-buffered saline, pH 7.4, containing 1 mM iodoacetic acid, 0.5 mM PMSF, 20 U/mL aprotinin and 2% of the non-ionic detergent TERIC (Sigma Chemical Co., St. Louis, USA). After homogenization, samples were centrifuged for 35 min at 51,000 g at 4°C in a Supraspeed RC28S Sorvall centrifuge (Sorvall Instruments, Du Pont Company, Wilmington, USA). The supernatants were collected and counted to calculate the percentage recovery of the original sample. All calculations accounted for this percentage recovery determined for each individual sample. Soluble TfR concentration was then measured on fresh supernatants of each homogenized organ.

Laboratory analyses

Blood samples were drawn from a tail vein. Part of the blood was drawn on ACD formula A and part on heparin to be centrifuged to obtain plasma that was frozen at -20°C until processing. Hematocrits were determined by the micromethod technique. Complete blood counts were measured on a Technicon H1 automatic cell counter (Tarrytown, New York, USA) with appropriate corrections for dilution by ACD. The percentage of reticulocytes was determined by cytofluorometry on a FACScan cytofluorometer (Becton Dickinson, San José, California, USA) after coloration with thiazole orange.[26] Preliminary experiments showed that this automated method gave results similar to manual counting on blood smears colored with brilliant cresyl blue. Soluble transferrin receptor (sTfR) was measured by ELISA in plasma as well as in organ extracts as previously described with minor modifications.[14,27] Serum iron (SeFe) and total iron binding capacity (TIBC) were measured by standard methods[28] and transferrin saturation was derived from these figures.

Calculation of the mass of TfR

The concentration of sTfR measured in the supernatant of each organ homogenate was corrected for the recovery of radioactivity counted in the original sample and expressed per mL (RBC and marrow) or g (liver and spleen) of organ. The total mass of TfR in each organ was then calculated based on this concentration and the total weight (liver and spleen) or volume (plasma, RBC and marrow) of the organ considered, taking into account the efficiency of organ extraction for the bone marrow, and multiplying the mass of the 2 femora by 6.5 to obtain the total TfR mass in the bone marrow. The total body mass of cellular TfR (*total TfR mass*) was then calculated as the sum of the total masses of TfR in liver, spleen, marrow, and RBC. The mass of RBC TfR was converted into number of TfR molecules per reticulocyte, using the following formula:

$$\text{N TfR/retic.} = \frac{\text{Total mass of RBC TfR } (\mu g)\times 10^{-6}\times 6.023\times 10^{23}}{190{,}000 \times \text{N retic. } (/\mu L)\times 70\mu L/g\times BW\ (g)}$$

where 6.023×10^{23} is Avogrado's number, 190,000 the molecular weight of TfR, and 70 µL/g the blood volume relative to body weight (BW).

Statistical methods

All results are presented as mean ± standard deviation (M ± SD) and expressed either in absolute values or as percentages of a baseline value. Comparisons of baseline values with later measurements in the same group of animals were carried out by paired Student's t tests. Comparisons between groups were carried out with Student's t-tests, with Welsch's correction in case of unequal variances. R correlation coefficients between two variables were computed in least squares regression equations. Most statistical analyses were carried out with Excel 97 (Microsoft Corporation, Redmond, Washington, USA) or Prism 2.0 (GraphPad Software Inc., San Diego, California, USA) software.

Table 1. Number of TfR molecules per reticulocyte in rats in various conditions not receiving iron or rHuEpo.

Condition	*Day*	*N*	*Tf saturation*	*Retic (%)*	*Retic (x10³/µL)*	*TfR (x 10³ molecules /retic)*
Normal	0	9	29±14	11.7±3.6	849.3±245.2	54.0±23.2
Hemolysis (PNH x 1)	2	4	31±10	13.9±2.8	839.0±133.4	72.0±11.6
	5	4	25±8	29.5±2.9#	1615.2±207.7#	120.5±23.9#
	10	4	28±8	40.7±8.4°	2719.9±614.3°	76.9±14.6
	16	8	25±8	8.4±1.3°	623.0±116.0*	36.0±11.9
Hemolysis (PNH x 3)	14	2	5±2°	42.5±2.6#	2355.1±115.2#	44.4±9.2
	18	2	6±0°	16.8±2.4	1203.0±313.2	63.5±1.6
Phlebotomy	5	5	5±2#	42.5±10.5°	1576.4±382.4#	68.2±6.4
	9	4	1±1#	34.6±1.9#	1651.6±74.3#	139.9±21.8#
	14	8	3±1#	41.6±6.0#	2024.6±315.4#	121.2±24.1#
Hypertransfusion (normal blood)	5	4	49±13*	2.5±0.8#	239.5±76.4#	35.5±13.2
	8	5	59±9°	1.0±0.3#	105.2±29.6#	52.5±22.6
Hypertransfusion (retic-rich blood)	5	4	82±9#	3.9±0.8#	327.8±65.9#	72.7±23.7
	11	4	83±11#	9.6±0.8	720.4±104.7	72.9±42.8
Hypertransfusion (retic-poor blood)	5	4	54±11°	0.7±0.1#	82.7±18.5#	41.6±7.1
	8	4	67±12#	1.0±0.4#	112.3±41.1#	54.9±29.2
	11	4	87±12#	1.0±0.5#	123.7±53.3#	69.6±20.4
	16	4	87±10#	0.8±0.3#	87.8±24.8#	33.7±12.5
Thiamphenicol (4 days)	4	4	44±5*	2.8±1.1#	203.1±85.8#	43.4±11.5
	7	3	8±3°	8.2±3.1	1215.4±245.1*	240.4±46.2#
	11	3	6±1°	13.1±1.3	807.7±98.9	68.3±16.7
Thiamphenicol (9 days)	10	3	–	1.5±0.0#	82.9±3.1#	54.4±11.7
Inflammation	1	4	9±2*	6.7±0.5°	449.9±25.6°	90.4±18.5*
	2	4	20±3	7.8±1.5	560.4±95.2*	70.3±24.6
	4	4	18±6	8.3±2.6	658.1±199.2	62.7±30.0

*PNH = phenylhydrazine. p values: *< 0.05, °<0.01, #<0.001 for comparison with normal animals. Retic = reticulocytes.*

Results

The number of TfR molecules per reticulocyte was measured in normal rats as well as in animals experiencing hemolysis, phlebotomies, hypertransfusions, thiamphenicol treatment or acute inflammation (Table 1). Normal rats had an average of 54,000 TfR molecules per reticulocyte and this did not vary significantly from the values found in most other groups of animals. However, after one week of phlebotomies, functional iron deficiency resulted in TfR numbers more than double normal values. Less severe degrees of functional iron deficiency did not result in such increased reticulocyte TfR numbers. TfR numbers were also increased on day 1 after injection of turpentine oil but normalized on day 2. Finally, in the early phase of erythroid regeneration after acute hemolysis, TfR expression was increased in reticulocytes. This was even more prominent in the recovery phase immediately after thiamphenicol-induced red cell aplasia.

The number of TfR molecules per reticulocyte was also investigated in relationship to age, iron status and rHu-Epo

Table 2. Number of TfR molecules per reticulocyte in normal, iron supplemented or iron overloaded rats of young or old age treated with rHuEpo.

Age group	Iron status	rHuEpo dose (U/d)	Day	N	Tf saturation (%)	Retic (%)	Retic (x10³/µL)	TfR (x10³ molecules /retic)
Young	Normal	0	0	6	18±2	6.4±2.8	543.7±199.8	67.1±17.0
		0	4	4	22±5	7.0±0.6	537.7±69.1	54.2±11.1
		0	8	4	16±5	10.0±2.4	777.5±152.7	54.2±29.4
		0	12	4	12±7	10.0±3.9	866.0±280.3	55.5±13.5
Young	Normal	10	4	4	15±1	11.3±4.2	906.2±299.4	55.2±17.4
		10	12	4	13±7	10.2±3.0	767.0±241.0	58.8±35.4
Young	Normal	50	4	4	6±3#	17.9±2.3#	1485.2±168.9#	103.1±52.3
		50	8	4	3±2#	12.8±0.9°	1159.2±67.4#	130.4±29.5*
		50	12	4	5±2#	16.9±3.9°	1626.7±333.0°	114.1±61.0
Young	Normal	150	4	4	2±1#	16.7±3.6°	1440.2±284.1°	115.3±17.9°
		150	8	4	3±0#	17.8±2.6#	1697.5±257.6#	141.1±32.8*
		150	12	4	1±1#	15.4±1.7#	1704.0±198.0#	163.8±53.5*
Young	Supplemented	0	0	6	26±2#	8.1±1.9	669.5±221.3	61.2±24.6
		0	12	4	21±1*	8.0±1.1	674.2±98.8	63.7±13.2
		50	12	4	17±6	17.9±0.4#	1783.7±57.3#	148.1±51.8*
		150	12	4	6±2#	19.1±1.9#	2037.5±236.0#	126.7±29.0*
Old	Normal	0	0	4	22±4	3.6±0.6	315.5±80.5	113.9±35.4
		0	16	4	20±6	3.0±0.5	281.7±40.6	105.6±32.0
Old	Overloaded	0	0	4	26±4	6.6±1.4*	570.7±116.3*	100.3±9.4
		10	16	4	20±3	7.6±0.7#	719.0±79.0#	113.5±25.1
		50	16	4	14±3*	10.1±1.2#	1047.7±125.1#	261.7±54.1°
		150	16	4	12±2*	12.5±1.8#	1357.7±220.2#	268.5±82.2*

*p values: *< 0.05; °<0.01; #<0.001 (young rats are compared with young normal animals receiving no rHuEpo, old rats with old normal rats receiving no rHuEpo). Retic = reticulocytes.*

Table 3. ETU and total mass of TfR in reticulocytes, marrow and spleen in rats transfused with normal, reticulocyte-poor and reticulocyte-rich blood and sacrificed on day 8.

	Normal rats	Rats hypertransfused with:		
		Normal blood	Retic-poor blood	Retic-rich blood
	N=9	N=5	N=4	N=4
Hct (%)	45.7±1.9	63.4±5.7°	66.3±2.2#	64.7±4.0#
Retic TfR (µg)	207±87	25±10#	30±13#	244±115
Marrow TfR (µg)	316±63	46±20#	60±9#	84±30#
Spleen TfR (µg)	17±16	2±1#	3±2#	9±9*
ETU (µMol/L/d)	88±33	16±6#	22±7#	40±6°

*p values: *< 0.05, ° <0.01, #<0.001 for comparison with normal animals. Retic = reticulocytes.*

treatment (Table 2). Older rats expressed more TfR in reticulocytes than younger animals. Iron supplementation for 2 weeks or iron overload for 3 months did not result in modifications of reticulocyte TfR numbers. Whereas low doses of rHuEpo (10 U/day) did not alter reticulocyte TfR expression, higher doses (50 to 150 U/day) resulted in significantly higher numbers of TfR but there was no difference between these latter two dose levels.

There was a strong correlation (r=0.60, p <0.001) between the fraction of injected radioactivity taken up by reticulocytes and their relative contribution to the total mass of TfR. However, in situations of intense stimulation of erythropoietic activity, reticulocyte radioiron uptake became much in excess of their relative TfR mass. For instance, whereas in normal rats 31±7% of the radioactivity went to reticulocytes (35±8% of total TfR mass) and 53±3% to the bone marrow (57±8% of total TfR mass), day 9 phlebotomized animals had 68±3% of radioactivity in reticulocytes (49±8% of total TfR mass) and 16±8% in the bone marrow (34±8% of total TfR mass).

Hypertransfusion with normal (Figure 1), reticulocyte-poor (Figure 2) or reticulocyte-rich (Figure 3) blood resulted in polycythemia-induced red cell aplasia. The total mass of marrow and spleen TfR was decreased (Table 3), demonstrating a severe reduction in the number of erythroblasts. Animals receiving normal or reticulocyte-poor blood had very low reticulocyte counts. Maximal suppression of erythropoietic activity was already achieved by day 5 but sTfR levels continued to decrease slowly and stabilized only later around 50% of baseline values when normal blood was transfused and 30% of baseline values when reticulocyte-poor blood was used. In rats transfused with reticulocyte-rich blood, reticulocyte counts first increased on day 1 before decreasing to levels that remained significantly higher than in the other groups. In contrast to the other animals, sTfR levels did not decrease but followed the evolution of reticulocyte counts. Despite adequate suppression of erythropoietic activity as documented by diminished ETU and a reduced mass of TfR in marrow and spleen (Table 3), sTfR levels remained consistently above baseline values (Figure 3). Indeed, whereas endogenous reticulocyte counts were probably considerably decreased, transfused reticulocytes maintained a normal total mass of reticulocyte TfR (Table 3). These donor

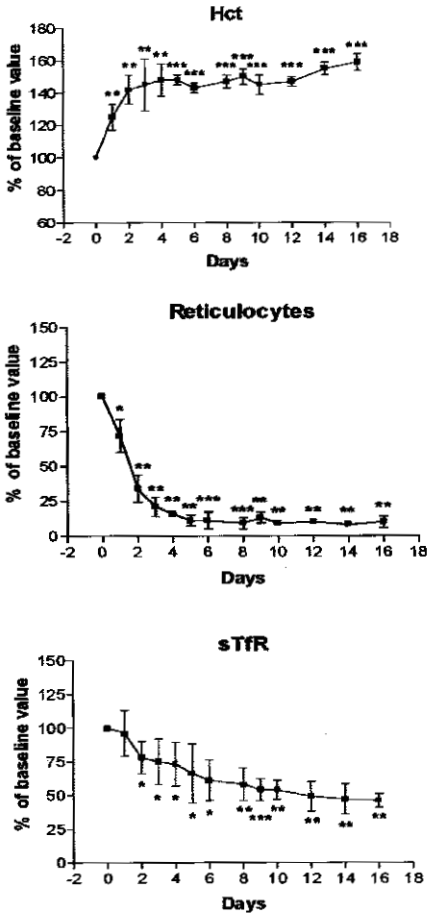

Figure 1. Evolution of the hematocrit, reticulocytes and sTfR levels in rats hypertransfused with normal blood.

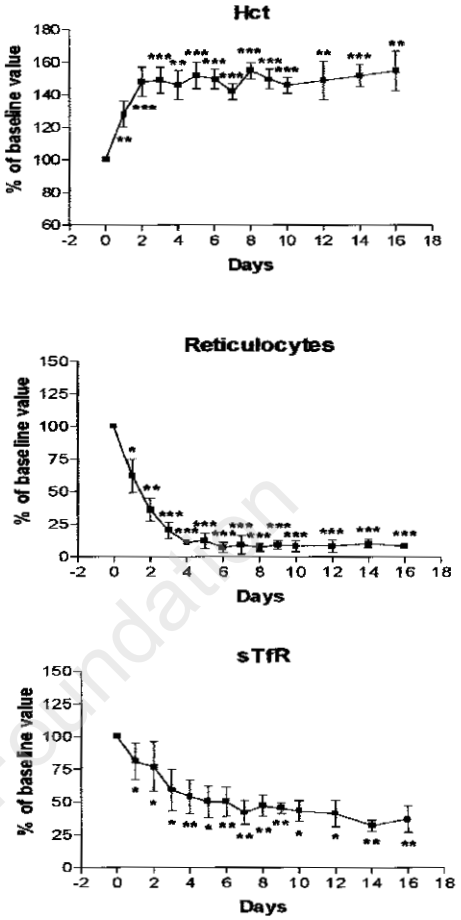

Figure 2. Evolution of the hematocrit, reticulocytes and sTfR levels in rats hypertransfused with reticulocyte-poor blood.

reticulocyte-associated TfR remained functional and took up adequate amounts of radioiron. Therefore, there was an excellent correlation between the total mass of erythron TfR and the ETU in the various groups of normal and hypertransfused animals (r=0.97, p =0.035).

Discussion

Although virtually all cells except mature red cells have TfR on their surface, the largest numbers are on the erythron. Previous estimates of erythroid cell TfR numbers were based on *in vitro* binding experiments of radioiodinated transferrin to erythroid cells. Many more receptors are seen on CFU-E than on BFU-E.[4-6] Assuming a unimolecular interaction between transferrin and TfR molecules in fetal liver erythroid cells, TfR numbers were evaluated to be about 300,000 on early normoblasts and up to 800,000 on intermediate normoblasts before declining to about 100,000 on reticulocytes.[7] TfR are lost during reticulocyte maturation so that TfR numbers vary with reticulocyte age and none is detected on mature red cells.[8,29,30] Using direct measurements we found TfR numbers in reticulocytes to be about half those previously reported. Similar[31] as well as lower[32] or higher[33] numbers of rat reticulocyte TfR have been reported by others. In another study on human reticulocytes, very low numbers of TfR (53±40 per cell) were found.[34] Others have demonstrated that there are fewer binding sites than immunoreactive TfR molecules.[29] However, some authors have emphasized the difficulty in estimating TfR numbers per reticulocyte in binding experiments since binding studies may vary according to the amount of transferrin present or the rate of intracellular internalization.[29,35] In addition, there is some controversy in the literature concerning binding relationships between TfR and transferrin, some assuming a 1:1 and others a 1:2 relationship.[1-3,33] If a 1:2 relationship is assumed, then previous estimates[7] should be halved and so become much closer to our figures. Finally, TfR numbers vary considerably with the age of the animals and this might account for some discrepancies

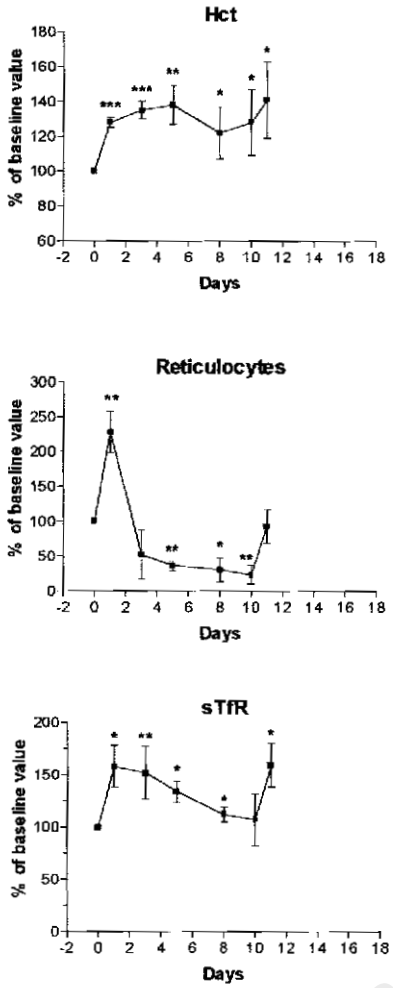

Figure 3. Evolution of the hematocrit, reticulocytes and sTfR levels in rats hypertransfused with reticulocyte-rich blood.

among studies. TfR expression was surprisingly higher in older animals, but as the number of reticulocytes was about half that in younger rats, the total mass of reticulocyte TfR was very similar. Although no such effect has been observed in human except in infants,[36,37] serum sTfR concentrations in rats have been reported to be lower in older animals.[14] However, the total mass of serum sTfR remains stable with age because the blood volume also increases with age.

TfR numbers were altered in a number of situations. Iron overload did not modify TfR expression in reticulocytes but this is not surprising because iron loading occurs mostly in non-erythroid cells. On the other hand, contrarily to previous reports,[32] iron deficiency expectedly induced higher TfR numbers but lower degrees of functional iron deficiency did not. The transient increase in TfR expression 24 hours after an episode of acute inflammation is more difficult to interpret. However, the total mass of reticulocyte TfR did not change significantly because the number of reticulocytes decreased. It has been previously reported that serum sTfR decreases transiently after acute inflammation.[14] This consistent early decrease of serum sTfR values without significant change in the mass of tissue TfR suggests that acute inflammation could accelerate the clearance of sTfR from the serum. However, it is also possible that lower serum sTfR values are only the result of an expansion of plasma volume mediated by cytokines such as interleukin-6.[38,39] Treatment with high doses of rHuEpo resulted in 2.5-fold higher numbers of TfR and this was relatively independent of functional iron deficiency because the increment was similar in normal, iron-supplemented and iron-overloaded animals that had very different transferrin saturations (Table 2). This is *in vivo* confirmation of the *in vitro* observation that Epo directly increases TfR expression in erythroid cells.[1,11] Finally, TfR expression was considerably increased in the early recovery phase after acute hemolysis or thiamphenicol-induced red cell aplasia but this occurred only transiently as the number of TfR per cell rapidly normalized despite ongoing reticulocytosis. Such an increase has been observed by others[32] and is consistent with younger reticulocytes shifting from the bone marrow to the circulation before a more mature population of cells follows suite. All these observations provide evidence that factors that have been shown to determine reticulocyte TfR expression in culture are also operational *in vivo*.

In situations of intense erythropoietic activity, radioiron uptake occurred preferentially in the large number of reticulocytes to the disadvantage of spleen or marrow erythroblasts, although they expressed comparable total numbers of TfR. It thus appears that, when iron is scarce relative to the demand for it, circulating reticulocytes more readily have access to transferrin iron and thus reinforce iron restriction in marrow or spleen erythroblasts. However, this aggravation of functional iron deficiency only occurs in the presence of massive reticulocytosis, a finding rarely encountered in humans.

In vitro incubation of reticulocytes[20,21] or erythroblasts[22] as well as various tumor cell lines[19,20,40] was followed by the release of TfR into the medium. Release of soluble TfR by erythroblasts occurred in the middle to late phase of maturation, the period of maximal mRNA and protein expression.[22] Exosomes containing intact TfR as well as truncated sTfR have been shown to be released by reticulocytes. Segregation of TfR and other obsolete proteins to a class of endosomes where fusion and budding occurs, results in the formation of multivesicular bodies that can fuse with plasma membrane and release exosomes into the circulation.[41-43] However, it has been shown that less than 1% of human serum TfR is intact receptor consistent with an exosomal origin, whereas virtually all is in the form of a soluble truncated extracellular domain.[44] Thus proteolysis occurs mostly at the surface of exosomes within the multivesicular body prior to exocytosis.[45] Re-incubation of externalized human exosomes failed to generate a soluble form of TfR.[45] However, other studies with rat reticulocytes have suggested that the production of sTfR was

still possible from externalized exosomes.[21]

In vivo, an excellent correlation between cellular TfR and soluble TfR has been demonstrated by ferrokinetic studies.[14,15] However, the cell population predominantly involved in TfR shedding has not been clearly identified. Based on a number of 5.3×10^9/kg normoblasts[46] and knowing that the vast majority of them are intermediate normoblasts expressing 800,000 TfR, the normoblast TfR mass can be estimated at 4.24×10^{15}/kg. This is almost four times more than the reticulocyte TfR cell mass of $1.13\text{x}10^{15}$/kg, based on a number of 8.2×10^9/kg marrow reticulocytes plus 3.1×10^9/kg blood reticulocytes[46] expressing 100,000 TfR. Therefore, the erythroblast compartment, not reticulocytes, should be the main source of sTfR. Indeed, serum sTfR levels parallel TfR expression on erythroblasts[47] and the highest reported levels were observed in thalassemic patients who are characterized by ineffective erythropoiesis with considerably expanded marrow erythropoiesis but low reticulocyte counts.[15] However, we show here that circulating reticulocytes can contribute considerably to serum sTfR levels , and this finding is in keeping with recent observations in athletes.[48] Despite severe erythroid aplasia induced by hypertransfusion, serum sTfR levels remained normal in rats repeatedly receiving reticulocyte-rich blood. This contrasted with the situation in rats transfused with normal or reticulocyte-poor blood in which sTfR values declined progressively to reach levels proportional to the number of circulating reticulocytes.

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ML conducted the experiments with rHuEpo while SR conducted the other studies, including the hypertransfusion experiments; YB designed and supervised the study and wrote the manuscript.

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Potential implications for clinical practice

Soluble tranferrin receptor levels are influenced not only by the mass of erythroblasts but also depend on transferrin receptor expression on reticulocytes.

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