Transferrin Reduces the Production of Soluble Transferrin Receptor (43904)

ROY D. BAYNES[1]

Division of Hematology, Department of Medicine, Kansas University Medical Center, Kansas City, Kansas 66103

Abstract. **The effect of homologous diferric transferrin from which contaminating transferrin receptor had been removed by monoclonal antibody affinity chromatography on soluble transferrin receptor concentrations was studied in K562 cells and HL60 cells in culture. Diferric transferrin in K562 cells caused a dose-dependent decrease in cellular receptor expression, a dose-dependent increase in cellular ferritin content, and a reduction in soluble receptor concentration which was of greater proportional magnitude than the reduction in cell receptor content. In HL60 cells, while there was a dose-dependent increase in cellular ferritin, cellular receptor content was relatively unaffected, while there was a consistent reduction in soluble receptor concentration. In both cells, the inhibitory effect of diferric transferrin on soluble receptor concentration was evident as early as 3 hr into the incubation. Apotransferrin, by contrast, did not reduce soluble receptor concentration. While elemental iron was capable of producing similar changes in cellular receptor and ferritin content, it had no inhibitory effect on proportional soluble receptor content. Studies employing other proteins, including human and bovine serum albumin, human lactoferrin, and rat ferritin, had no inhibitory effect on soluble receptors concentration, thus confirming the specificity of the findings. Control studies excluded an assay artifact as the explanation for the current findings. Prior contrary reports appear completely explained by the combination of soluble transferrin receptor contaminating the transferrin employed for study and a systematic difference in the assays employed between free and transferrin-bound receptor.** [P.S.E.B.M. 1995, Vol 209]

The importance of the transferrin receptor for cellular iron procurement, cellular growth, and replication has long been appreciated (1, 2). A soluble form of receptor is uniformly present in human sera (3–5). It has already realized major significance as a sensitive and specific indicator of tissue iron deficiency (6), as a noninvasive measure of total cellular transferrin receptor mass (7), the single best indicator of tissue iron deficiency in pregnancy (8) and the only noninvasive test to reliably distinguish iron deficiency anemia from the anemia of chronic disease (9). This serum form of receptor has been biochemically defined as a truncated monomer with the site of truncation in the extracellular domain just adjacent the cell membrane (10, 11). Production of an identical soluble form of receptor has been reported by cell lines in culture (12, 13). Work from this laboratory has established that the serum receptor is produced by proteolysis predominantly at the surface of the exosome and possibly to a minor extent at the cell surface (14, 15). A membrane-associated serine protease that is responsible for cleavage has been identified (16). Given the cellular receptor's high affinity for diferric transferrin and the subsequent process of receptor-mediated endocytosis, the effect of diferric transferrin on the production of the soluble receptor by cultured cells needs to be defined. Since increased cellular iron delivery results from endocytosis of transferrin iron and since post-transcriptional downregulation of receptor expression might be anticipated (17), relating changes in soluble receptor to changes in cellular receptor content is important. Conflicting data have, however, been obtained. Study of K562 cells using an ELISA

[1] To whom requests for reprints should be addressed at Division of Hematology, Kansas University Medical Center, 39th & Rainbow, Kansas City, KS 66103.

Received September 1, 1994. [P.S.E.B.M. 1995, Vol 209]
Accepted February 9, 1995.

0037-9727/95/2093-0286$10.50/0

based on OKT9 and B3/25 monoclonal antibodies along with in-house-purified transferrin reported that this ligand markedly enhanced soluble receptor production (18). This suggested that the process of soluble receptor production was possibly endocytosis related. Assessment of HL60 cells using a semi-quantitative dot blot analysis with OKT9 monoclonal antibody and commercial transferrin reported no effect of ligand on soluble receptor production (19). This suggested that the process was ligand independent. The current investigation evaluated this further by employing immunological reagents developed in this laboratory (5) along with immunoaffinity removal of transferrin receptor activity (10) from transferrin preparations together with our previously reported K562 system (12, 13). HL60 leukemic cells were also studied. The findings were that all preparations of transferrin studied were contaminated with significant amounts of soluble receptor. This was electrophoretically identical with the serum form of transferrin receptor. When diferric transferrin from which receptor activity had been removed was studied, it appeared to limit soluble receptor concentration in both cell lines. The reduction in soluble receptor concentration exceeded any reduction in cellular receptor content. This effect was specific to transferrin and not related to enhanced iron supply.

Materials and Methods

Materials. Diethanolamine, Tween 80, Tris, Teric (polyoxyethylene 9 lauryl ether), RPMI 1640, pyruvate, glutamine, transferrin, bovine serum albumin, human serum albumin, and cyanogen bromide-activated Sepharose 4B were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum was obtained from Hazelton Research Products (Denver, CO). Rat ferritin was prepared from iron-loaded rat livers and human lactoferrin from human breast milk. A tissue culture antibiotic (penicillin and streptomycin) and antifungal (amphotericin) preparation was obtained from GIBCO (Grand Island, NY). Ferric ammonium citrate was obtained from Fisher (St. Louis, MO). Culture test tubes and disposable sterile pipettes were obtained from Falcon, Becton Dickinson (Lincoln Park, NJ), and culture flasks were obtained from Corning (Corning, NY).

Biochemical Procedures. Affinity removal of transferrin receptor from solutions containing transferrin by means of anti-transferrin receptor monoclonal antibody derivatized sepharose beads, as well as the elution procedure, SDS-PAGE, and staining were as previously described (10). Apotransferrin was saturated with iron by the method of Edwards and colleagues (20).

Immunoassay Procedure. An enzyme-linked immunoassay (ELISA) was employed to measure the concentration of soluble receptor in culture supernatants. This assay was established with monoclonal antibodies prepared against transferrin saturated receptor isolated from human placenta (5). The ELISA protocol was as previously described except that 200 μl of supernatant was measured directly and solubilized cell pellet was diluted 1/20 directly in the well with assay buffer rather than with any prior dilution (13). The assay was standardized against purified transferrin-receptor complex, which contained 54% transferrin receptor as determined by protein and ELISA measurements on unbound placenta receptor. All assays were performed in triplicate. Studies in which transferrin receptor standard was added to culture medium gave recoveries of 107%–114% (12, 13). Cellular receptor content and ferritin concentration were measured after overnight solubilization of cells in 1% teric as previously described (12, 13). Transferrin content of serum-free media was evaluated by a monoclonal antibody ELISA as previously described (21). All proteins studied were assayed to exclude transferrin receptor contamination.

Cell Culture. K562 erythroleukemic cells were maintained in log phase growth in RPMI 1640 medium which was supplemented with glutamine, pyruvate, and the antibiotic/antimycotic solution. The basic culture medium was further enriched with 10% fetal calf serum. Cells were cultured in a 5% CO_2 environment at 37°C. HL60 cells were maintained under similar conditions except that the medium was enriched with 20% fetal calf serum.

In individual experiments 1×10^6 cells suspended in 1 ml of serum-free medium were placed in 5 ml culture tubes and incubated, unless otherwise indicated, for 24 hr. The different conditions evaluated included the addition of various concentrations of affinity purified apo- and diferric transferrin, nonpurified diferric transferrin, ferric ammonium citrate, bovine serum albumin, human serum albumin, rat ferritin, and human lactoferrin. Viability of cultures was assessed by trypan blue exclusion which exceeded 97.5% in all studies.

After 24-hr incubation, the tissue culture tubes were placed on a level oscillator for 2 min to obtain an even cell suspension. A small aliquot (40 μl) was removed for measurement of cell number using an electronic particle counter (ZBI; Coulter Electronics, Hialeah, FL). After centrifuging each tube for 10 min at 2000*g* the supernatant was decanted into a 2-ml microfuge tube and immediately frozen. The remaining pellet was suspended in 1 ml Hank's buffered salt solution containing 0.001 *M* Tris and 1% Teric, pH 7.4 at 4°C. This was then agitated overnight at 4°C on an end-over-end rotator and then centrifuged at 4°C at 15000*g* for 15 min. The decanted supernatant was frozen at −20°C until assayed. All values reported rep-

resent the results obtained in at least four separate incubations.

Statistical Comparisons. Within and between group comparisons were by analysis of variance.

Results

The initial evaluation involved the measurement of transferrin receptor activity by ELISA in solutions made up from commercial transferrin in RPMI 1640 medium. All preparations evaluated contained significant receptor activity, which, for the commercial transferrin currently employed, was quantitatively on the order of 0.02% of the transferrin by weight. No transferrin receptor activity could be detected after transferrin was passed through an affinity column prepared with monoclonal antibody to the intact transferrin receptor. This transferrin receptor-free transferrin was employed in the subsequent evaluations.

The response of the two cell lines to incubation in the presence of increasing amounts of diferric transferrin are shown after 24 hr of incubation in Table I. The K562 cells showed a progressive decline in cellular receptor content with increasing concentrations of diferric transferrin such that in the presence of 4000 pmole/ml iron as diferric transferrin there was a 40% reduction in transferrin receptor content. This was accompanied by a 250% increase in cellular ferritin. The soluble receptor showed a more marked 50% reduction such that the proportion of soluble to cellular receptor showed a 17% reduction. The HL60 cells appeared to have a lower cellular receptor content, which changed little in response to the added transferrin despite a 328% increase in cellular ferritin content. The soluble receptor, by contrast, showed a 26% reduction in the presence of 4000 pmole/ml iron as diferric transferrin such that the proportion of soluble to cellular receptor showed a 22% reduction. When the cellular ferritin content and the supernatant to cellular transferrin receptor ratio are calculated as a percentage of the values obtained in the absence of added diferric transferrin (Fig. 1), it can be appreciated that the responses to diferric transferrin were very similar in the two cell lines in terms of reducing soluble receptor concentration.

Time-dependent studies were conducted in which K562 cells and HL60 cells were incubated in the absence or presence of 1320 pmole iron/ml as diferric transferrin. This was done because certain of the prior data had employed briefer incubation periods (19) and longitudinal data might provide information on the relationship between iron supply and reduction in soluble receptor production. These data are summarized in Table II.The K562 cells showed a 17%, 5%, and 25% reduction in cell receptor content after 3, 6, and 22 hr of incubation, respectively, which was accompanied by a 37%, 71%, and 129% increase in cellular ferritin content. By contrast, there was a 44%, 46%, and 42% reduction in soluble receptor concentration at the various times such that the proportion of soluble to cellular receptor was reduced by 36%, 40%, and 24%, respectively. The HL60 cells in culture showed approximately a 40% reduction in cellular receptor content in the presence or absence of the diferric transferrin over 22 hr of incubation. There was, however, no difference in cell receptor content between growth in the presence or absence of diferric transferrin at any time. This was despite a progressive increase in cellular ferritin of 48%, 106%, and 274% at 3, 6, and 22 hr. Soluble receptor showed a relatively consistent 31%, 36%, and 34% reduction at the times studied such that

Table I. The Dose-Dependent Effect of Homologous Diferric Transferrin on Cellular Receptor Content, Soluble Receptor Concentration, the Proportional Soluble Receptor Content and Cellular Ferritin After 24-hr Incubation

Iron as diferric transferrin (pmole/ml)	Cellular receptor		Cellular ferritin[a]		Soluble receptor		Proportional soluble receptor	
	K562 (fg/cell)	HL60 (fg/cell)	K562 (fg/cell)	HL60 (fg/cell)	K562 (fg/cell)	HL60 (fg/cell)	K562 (%)	HL60 (%)
0	297 (10)	110 (4)	18 (17–19)	25 (24–27)	12 (0)	42 (2)	4.1 (0.2)	38.2 (0.4)
2	297 (24)	103 (9)	18 (16–20)	26 (24–28)	12 (1)	43 (3)	4.0 (0.2)	42.9 (2.6)
5	288 (11)	109 (11)	20 (19–21)	31 (30–32)	12 (1)	40 (1)	4.3 (0.4)	36.8 (4.4)
10	258 (18)	100 (11)	21 (20–23)	34 (33–35)	11 (1)	38 (2)	4.3 (0.4)	38.0 (3.8)
20	254 (28)	104 (15)	24 (24–25)	39 (37–41)	11 (0)	38 (2)	4.2 (0.3)	37.1 (5.2)
100	207 (11)	122 (7)	33 (32–34)	56 (51–60)	8 (0)	38 (5)	4.0 (0.2)	31.0 (3.7)
200	186 (15)	107 (15)	38 (34–42)	61 (59–63)	8 (0)	33 (2)	4.3 (0.4)	31.3 (3.6)
1000	177 (22)	117 (14)	57 (52–63)	79 (71–87)	6 (0)	34 (3)	3.4 (0.2)	29.1 (1.6)
2000	178 (12)	116 (6)	62 (55–63)	90 (84–97)	6 (0)	35 (2)	3.6 (0.1)	30.7 (0.4)
4000	177 (12)	106 (13)	63 (60–67)	107 (100–115)	6 (0)	31 (1)	3.4 (0.1)	29.7 (4.0)
f value	35.3	1.6	152.9	270.4	110.8	9.4	6.1	7.6
P value <	0.0001	0.02	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Note. Values shown as mean and 2SE. Statistical intragroup comparison by analysis of variance is shown.
[a] Geometric mean and 2SE range.

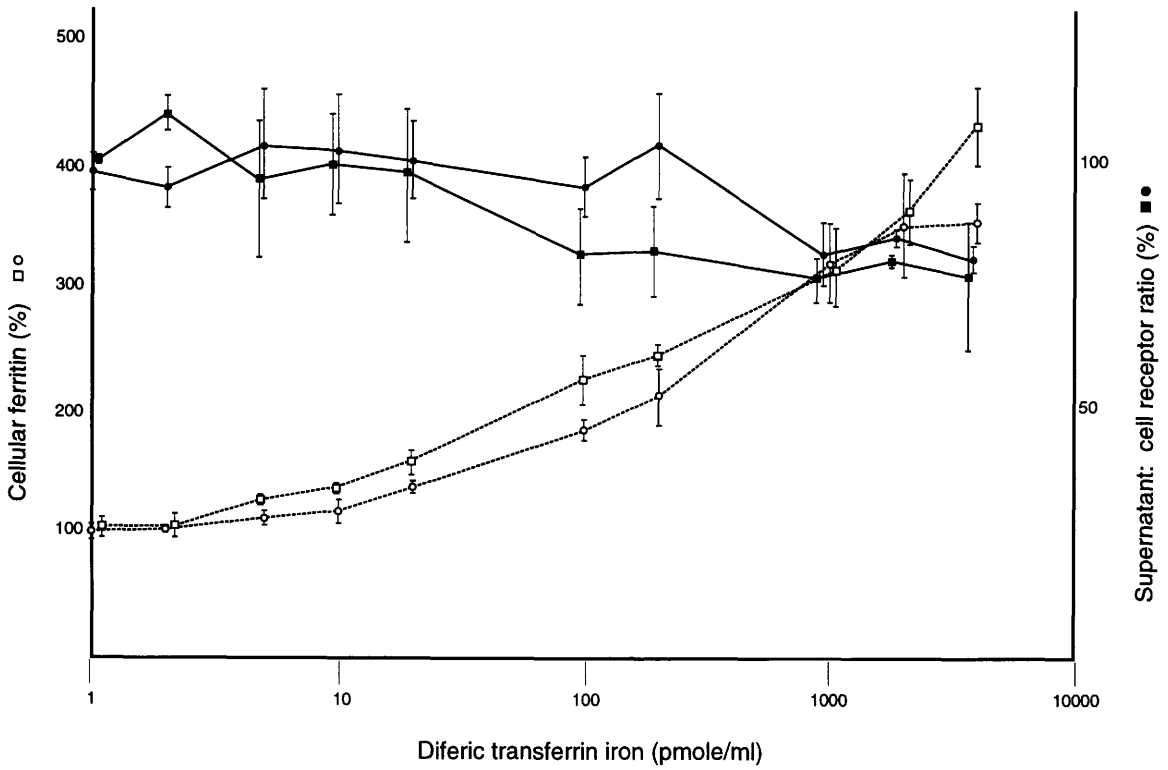

Figure 1. Dose-dependent changes in cellular ferritin content (open) and the supernatant: cellular transferrin receptor ratio (solid) for K562 cells (circles) and HL60 cells (squares). Values were calculated as a percentage of the values obtained in the absence of added diferric transferrin. Mean ± 2SE values are shown.

Table II. Time-Dependent Effect of Homologous Diferric Transferrin (1320 pmole/ml Iron as Diferric Transferrin) on Cellular Receptor Content, Soluble Receptor Concentration, the Proportional Soluble Receptor Content, and Cellular Ferritin

Duration of incubation	Cellular receptor		Cellular ferritin[a]		Soluble receptor		Proportional soluble receptor	
	K562 (fg/cell)	HL60 (fg/cell)	K562 (fg/cell)	HL60 (fg/cell)	K562 (fg/cell)	HL60 (fg/cell)	K562 (%)	HL60 (%)
3 hr								
−Transferrin	250 (16)	206 (12)	14.4 (13.5–15.4)	16.0 (15.5–16.6)	6.1 (0.2)	30.6 (2.1)	2.5 (0.2)	14.9 (1.6)
+Transferrin	207[b] (17)	210 (42)	19.7[b] (18.6–20.9)	23.6[b] (22.4–24.7)	3.4[b] (0.2)	21.0[b] (1.0)	1.6[b] (0.1)	10.4[c] (2.6)
6 hr								
−Transferrin	246 (17)	183 (9)	13.6 (12.8–14.4)	18.5 (18.0–19.1)	7.4 (0.7)	42.1 (3.8)	3.0 (0.3)	23.0 (0.9)
+Transferrin	223 (16)	203 (30)	23.3[b] (21.7–25.5)	38.2[b] (32.4–45.1)	4.0[b] (0.4)	27.0[b] (1.3)	1.8[b] (0.1)	13.6[b] (2.0)
22 hr								
−Transferrin	249 (21)	118 (12)	11.9 (11.6–12.1)	24.4 (22.5–26.4)	9.2 (0.2)	63.7 (6.8)	3.7 (0.3)	54.2 (4.0)
+Transferrin	188[b] (14)	129 (10)	27.2[b] (25.4–29.1)	91.3[b] (85.5–97.5)	5.3[b] (0.6)	42.2[b] (4.0)	2.8[b] (0.1)	33.0[b] (4.0)

Note. Mean and 2SE values are indicated.
[a] Geometric mean and 2SE range.
[b] Significantly different from nontransferrin incubations ($P < 0.005$).
[c] Significantly different from nontransferrin incubation ($P < 0.05$).

the proportion of soluble to cell receptor was consistently reduced by 30%, 41%, and 39%, respectively, in the presence of diferric transferrin. These data indicate that the inhibitory effect of diferric transferrin on soluble receptor proportional concentration is established early and is relatively constant despite a progressive increase in cellular iron content as reflected in the cellular ferritin content.

The effect of iron supply, *per se*, on soluble receptor production was addressed by the study of cells cultured in the presence of elemental iron provided as ferric ammonium citrate. The findings are summarized in Table III. The HL60 cells appeared to readily assimilate the elemental iron. This is reflected by a progressive increase in cellular ferritin content reaching a 233% increase in the presence of 4000 pmole/ml iron. Cellular receptor was, however, virtually unchanged. The increased cellular iron content was not associated with any decrease in proportional soluble receptor concentration. The K562 cells, by contrast, were unable to use the elemental iron as readily as were the HL60 cells. To overcome this relative resistance, more iron-deficient cells were prepared by use of only 5% fetal calf serum supplemented medium and higher concentrations of ferric ammonium citrate were utilized. Using this approach, a progressive decrease in cellular transferrin receptor was associated with a progressive increase in cellular ferritin such that at a concentration of 200,000 pmole/ml iron, cellular receptor was reduced by 45% and cellular ferritin increased by 294%. Soluble receptor showed a parallel reduction which proportionally was slightly less than the reduction in cellular receptor content such that the proportion of soluble to cellular receptor showed no reduction and if anything a slight increase. The changes in cellular ferritin and the supernatant cellular receptor ratio expressed as a percentage of the control incubations are shown on Figure 2. These data indicate clearly that the greater proportional inhibitory effect of diferric transferrin on soluble receptor concentration is not a reflection of enhanced iron supply but relates to an effect of the ligand.

The effect of the addition of diferric transferrin to free soluble transferrin receptor in culture medium was evaluated to exclude the possibility that the observed inhibitory influence of transferrin on soluble receptor concentrations might represent a systematic difference in the assay detection of free and bound soluble transferrin receptor. Ligand free soluble transferrin receptor was obtained from human serum as previously described (10). Incremental amounts of diferric transferrin were added to a constant amount of ligand free soluble receptor in culture medium. Binding of transferrin to the soluble receptor was confirmed by a shift in the receptor peak as determined by ELISA on HPLC gel filtration separated fractions. The results of these additional studies are summarized in Table IV. It can be seen that over a wide molar excess range of purified diferric transferrin there was no variation in soluble receptor detection.

The question of whether the inhibitory effect might represent a nonspecific one in which protein acts as a competitive protease inhibitor was addressed. To this end, the effects of other proteins including bovine serum albumin, human serum albumin, rat ferritin, and human lactoferrin were added to cultures at concentrations comparable to those employed in the transferrin evaluations (25 and 75 μg/ml). The

Table III. The Dose-Dependent Effect of Iron Added as Ferric Ammonium Citrate on Cellular Receptor Content, Soluble Receptor Concentration, the Proportional Soluble Receptor Content, and Cellular Ferritin After 30 hr of Incubation

K562 Cells					HL60 Cells				
Iron as ferric ammonium citrate (pmole/ml)	Cellular receptor (fg/cell)	Cellular ferritin (fg/cell)	Soluble receptor (fg/cell)	Proportional soluble receptor (%)	Iron as ferric ammonium citrate (pmole/ml)	Cellular receptor (fg/cell)	Cellular ferritin (fg/cell)	Soluble receptor (fg/cell)	Proportional soluble receptor (%)
0	591	11.1	34.1	5.8	0	106	56.5	65.8	62.7
	(25)	(9.9–12.3)	(2.1)	(0.3)		(10)	(54.7–58.3)	(4.0)	(7.0)
100	605	10.9	33.0	5.5	2	109	53.0	66.0	60.8
	(38)	(9.1–12.7)	(0.6)	(0.4)		(6)	(48.3–58.3)	(4.0)	(7.0)
200	528	9.6	31.9	6.0	5	111	51.0	70.1	63.8
	(18)	(8.4–10.8)	(1.6)	(0.3)		(19)	(45.8–56.9)	(4.8)	(6.6)
1000	583	10.6	33.8	5.8	10	109	56.5	64.5	60.2
	(24)	(8.8–12.4)	(1.4)	(0.1)		(14)	(51.7–61.7)	(1.4)	(8.1)
2000	578	12.0	35.3	6.2	20	110	58.1	68.1	62.1
	(41)	(10.2–13.8)	(2.4)	(0.7)		(10)	(50.0–67.4)	(2.4)	(4.0)
10,000	481	15.3	29.2	6.1	100	118	70.8	65.3	55.5
	(32)	(13.7–16.9)	(0.7)	(0.3)		(8)	(69.3–72.3)	(5.4)	(6.1)
20,000	430	21.5	24.5	5.7	200	101	93.1	71.7	70.6
	(22)	(18.9–24.1)	(0.8)	(0.3)		(2)	(89.0–97.4)	(2.8)	(2.6)
40,000	359	29.6	24.0	6.7	1000	98	127.0	59.8	61.4
	(17)	(28.2–31.0)	(2.2)	(0.4)		(10)	(119.5–135.0)	(4.0)	(8.8)
100,000	356	33.8	23.3	6.6	2000	102	141.8	56.4	55.1
	(22)	(32.0–35.6)	(1.8)	(0.3)		(2)	(131.0–153.5)	(3.6)	(3.6)
200,000	325	43.7	22.3	6.9	4000	89	188.1	59.7	66.8
	(22)	(40.1–47.3)	(1.4)	(0.2)		(7)	(173.1–200.3)	(3.2)	(4.4)
f value	39.9	73.6	31.8	6.2		2.4	125.9	6.4	1.9
P value <	0.0001	0.0001	0.0001	0.0001		0.04	0.0001	0.0001	0.06

Note. Values are shown as mean and 2SE. Statistical intragroup comparisons by analysis of variance are shown.

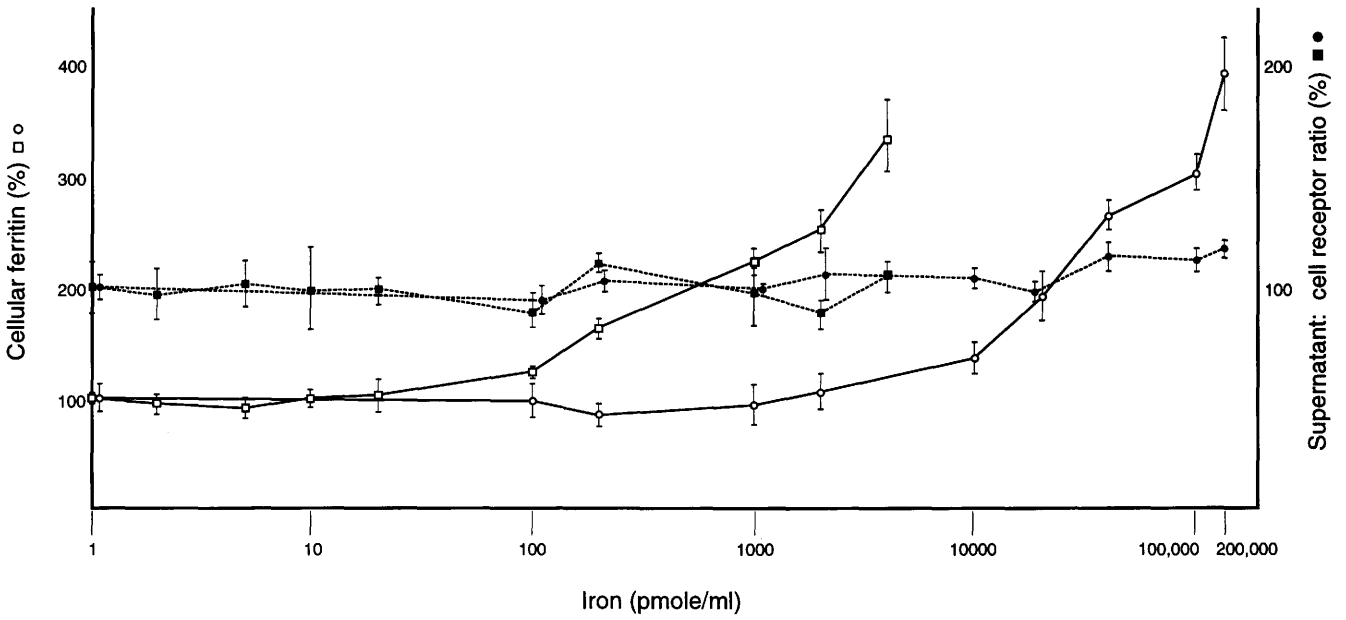

Figure 2. Dose-dependent changes in response to non transferrin iron in cellular ferritin content (open) and the supernatant: cellular transferrin receptor ratio (solid) for K562 cells (circles and HL60 cells (squares). Values were calculated as a percentage of the values obtained in the absence of added ferric ammonium citrate. Mean ± 2SE values are shown.

Table IV. The Absence of a Ligand-Dependent Effect on Receptor Measurement

Amount of soluble receptor (ng)	Amount of added transferrin (ng)	Value of receptor assayed (ng)	Percentage assayed (%)
156.5	17.5	151.5	96.8
156.5	62.5	152.9	97.7
156.5	350.0	158.9	101.5
156.5	700.0	164.3	104.9

Note. Incremental amounts of diferric transferrin were added to a constant amount of ligand free soluble transferrin receptor and receptor activity measured by the ELISA. Binding was confirmed by HPLC gel filtration analysis.

effect of these proteins on the proportion of supernatant to cellular receptor in cultures of K562 and HL60 cells is summarized in Table V. In both cells lines and at both concentrations, none of the proteins produced any significant reduction in proportional soluble receptor concentration.

A final experiment evaluated the effect of apotransferrin on soluble receptor concentration (Table VI) in K562 cells cultures. In this study, a progressive reduction in cell receptor concentration reaching a maximum of approximately 31% can be seen. This is accompanied by a 27% reduction in soluble receptor along with an approximately 80% increase in cellular ferritin content. However, there is no reduction in the ratio of soluble to cellular receptor. These data are best explained by apo-transferrin binding small amounts of iron in the culture system and making this available to cells but at a concentration of iron bound transferrin insufficient to modify soluble receptor production.

Discussion

The current investigation has defined the effect of diferric transferrin on the concentration of soluble transferrin receptor, which is in all likelihood reflective of soluble receptor production by cells in culture. When diferric transferrin from which intrinsic contaminant transferrin receptor has been removed is added to either K562 or HL60 cells in culture, it results in a decrease in supernatant transferrin receptor content of greater magnitude than any reduction in cellular receptor content. This greater reduction is not an artifact due to reduced detection of the complex of receptor and transferrin in the ELISA system used. Prior work employing intact receptor (5) and current data employing ligand free soluble receptor indicate that the ELISA system used detects free and bound receptor equally. Studies employing nontransferrin proteins including bovine serum albumin, human serum albumin, rat ferritin, and human lactoferrin showed no inhibition of soluble receptor production. These data indicate that the effect is transferrin specific and not due to competitive inhibition of proteolysis. The fact that purified apotransferrin had no effect on proportional soluble transferrin receptor concentration indicates that the effect is diferric-transferrin specific. This, in accordance with the well-documented significantly higher affinity of receptor for diferric transferrin, indicates that the effect is dependent on receptor ligand interaction. Time-dependent studies showing constant

Table V. The Absence of an Inhibitory Effect of Other Proteins on Soluble Receptor Production by K562 and HL60 Cells

Protein	Percentage soluble receptor			
	K562 Cells		HL60 Cells	
	25 μg/ml protein	75 μg/ml protein	25 μg/ml protein	75 μg/ml protein
Control (no protein added)	4.8 (0.4)	4.8 (0.4)	59.2 (3.6)	59.2 (3.6)
Bovine serum albumin	4.8 (0.4)	4.7 (0.2)	57.0 (1.0)	60.0 (4.8)
Human serum albumin	5.1 (0.3)	5.0 (0.1)	51.7 (2.8)	59.4 (5.2)
Rat ferritin	5.8[a] (0.4)	6.3[a] (0.3)	54.9 (3.4)	60.0 (4.8)
Human lactoferrin	4.6 (0.2)	4.7 (0.1)	55.6 (3.8)	58.4 (3.0)

Note. Mean ± 2SE values are indicated. Cells were incubated for 24 hr.
[a] Significantly increased compared with controls ($P < 0.05$).

inhibition of soluble receptor production despite increasing cellular iron content in the presence of diferric transferrin, along with a lack of inhibition of soluble receptor production when cellular iron content is comparably increased with non-transferrin iron, indicate that the transferrin effect of reducing soluble receptor production is not related to iron supply. While the reduction in cellular receptor content occurs comparably with transferrin and nontransferrin iron, the greater reduction of supernatant occurs only with diferric transferrin.

There are a number of possible mechanisms whereby diferric transferrin might reduce soluble receptor production. One possibility is the well-known effect of diferric transferrin in enhancing receptor endocytosis, thus effecting a redistribution of cellular receptor from surface to the cellular interior (22). This redistribution, together with the suggestion that soluble receptor might be generated at the cell surface by proteolysis (23), fits in well with the current observations. Our data indicating that receptor proteolysis tends to occur more completely at the exosome surface than at the cell surface (14, 15) raise the second possibility that the intracellular handling of endocytosed receptor may vary significantly depending on occupancy by transferrin (24). Another, not mutually exclusive possibility is that intact receptor bound to transferrin might be resistant to proteolysis. A fourth, less likely possibility is that a soluble receptor-transferrin complex might be more susceptible to further degradation with loss of immunoactivity. While there are a number of possible mechanisms to explain the observation, it does have major value in excluding endocytosis of occupied receptor as the mechanism of soluble receptor production (18).

The findings reported in the current study, namely that homologous diferric transferrin inhibits soluble transferrin receptor production, are clearly contrary to the two previous studies evaluating this question (18, 19). One factor contributing to the prior findings is

Table VI. The Dose-Dependent Effect of Homologous Apotransferrin on Cellular Receptor Content, Soluble Receptor Concentration, the Proportional Soluble Receptor Content and Cellular Ferritin in K562 Cells After 24-hr Incubation

Apotransferrin (μg/ml)	Cellular receptor	Cellular ferritin[a]	Soluble receptor	Proportional soluble receptor
0.0	278 (18)	15 (13–18)	11 (1)	4.1 (0.3)
0.075	266 (6)	18 (17–19)	12 (1)	4.4 (0.6)
0.188	261 (25)	19 (18–20)	11 (1)	4.1 (0.4)
0.375	241 (20)	19 (18–21)	11 (1)	4.6 (0.4)
0.750	215 (11)	21 (19–23)	10 (0)	4.5 (0.2)
3.750	210 (14)	27 (26–28)	9 (1)	4.2 (0.5)
7.500	191 (12)	22 (19–26)	8 (0)	4.3 (0.3)
37.500	192 (22)	27 (25–30)	9 (1)	4.8 (0.4)
75.000	205 (18)	26 (25–27)	9 (0)	4.5 (0.2)
150.000	209 (12)	24 (23–24)	9 (0)	4.3 (0.2)
f value	15.1	19.3	11.3	1.4
P value <	0.0001	0.0001	0.0001	0.3

Note. Values shown as mean and 2SE. Intragroup statistical comparisons by analysis of variance are shown.
[a] Geometric mean and 2SE range.

likely to have been the contamination of purified transferrin with soluble transferrin receptor. This contaminant activity could be completely removed by monoclonal antibody immunoaffinity chromatography. Interestingly, when the immunoaffinity column was eluted (10) and the eluate subjected to SDS-PAGE under nonreducing conditions two peptides of 75K and 85K were observed. These were confirmed as having previously been identified as transferrin and soluble receptor, respectively (10). In the case of the Kohgo study (18), at the concentration of diferric transferrin employed (100 μg/ml), if the level of contamination with the receptor was comparable to what we have documented, an enhancing effect would be anticipated. However, these workers reported a progressive increase in soluble receptor over time compared with controls. If the increase merely reflected the contaminant soluble receptor, it would be expected that, over time, the apparent increase would be constant. The explanation for the apparent increase relates, in all likelihood, to the OKT9 and B3/25 assay system. Prior work has shown that there is a systematic difference with this assay between free- and bound-soluble receptor in favor of the free form (5). Consequently, it would appear likely that, as iron is taken up into cells over time, more apotransferrin is produced which has lower affinity for receptor thereby displacing the equilibrium in favor of free receptor. This would result in an apparent progressive increase in soluble receptor production. In the case of the Chitambar study (19), which employed the same transferrin source as in the current investigation at concentrations of 5 μg/ml and 50 μg/ml, not unexpectedly, no inhibitory effect was noted. Indeed, the question then arises of why the contaminant did not give the appearance of increased production. In part, the dot blot shown in that study did appear to demonstrate an increase at the 50 μg/ml concentration. The indicator system employed the OKT9 antibody and consequently the comments in relation to the Kohgo study concerning a systematic difference between free and bound receptor again apply. Given the mild reduction in cell receptor noted in the current study even after 3 hr, some minor decrease in soluble receptor may have been expected even if cellular receptor production were unaffected.

The current finding of inhibition of soluble receptor concentrations in cell cultures in the presence of homologous diferric transferrin is of importance because it corrects the prior incorrect notions that production was either dependent on ligand (18) or not inhibited by ligand (19). The specific level at which diferric transferrin exerts this ligand-specific inhibitory effect is not known but its elucidation will help refine our understanding of the mechanisms of soluble receptor production.

The author acknowledges, with thanks, the technical assistance of Lilla Khalifah and the helpful comments of Dr. James Cook.

1. Morgan EH. Transferrin: Biochemistry, physiology and clinical significance. Mol Aspect Med **4**:1–123, 1981.
2. Huebers HA, Finch CA. The physiology of transferrin and transferrin receptors. Physiol Rev **67**:520–582, 1987.
3. Kohgo Y, Nishisato T, Kondo H, Tsushima N, Niitsu Y, Urushizaki I. Circulating transferrin receptor in human serum. Br J Haematol **64**:277–281, 1986.
4. Kohgo Y, Niitsu Y, Kondo H, Kato J, Tsushima N, Sasaki K, Hirayama M, Numata T, Nishisato T, Urushizaki I. Serum transferrin receptor as a new index of erythropoiesis. Blood **70**:1955–1958, 1987.
5. Flowers CH, Skikne BS, Covell AM, Cook JD. The clinical measurement of serum transferrin receptor. J Lab Clin Med **114**:368–377, 1989.
6. Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: A quantitative measure of tissue iron deficiency. Blood **75**:1870–1876, 1990.
7. Huebers HA, Beguin Y, Pootrakul P, Einspahr D, Finch CA. Intact transferrin receptors in human plasma and their relation to erythropoiesis. Blood **75**:102–107, 1990.
8. Carriaga MT, Skikne BS, Finley B, Cutler B, Cook JD. Serum transferrin receptor for the detection of iron deficiency in pregnancy. Am J Clin Nutr **54**:1077–1081, 1991.
9. Ferguson BJ, Skikne BS, Simpson KM, Baynes RD, Cook JD. Serum transferrin receptor distinguishes the anemia of chronic disease from iron deficiency anemia. J Lab Clin Med **119**:385–390, 1992.
10. Shih YJ, Baynes RD, Hudson BG, Flowers CH, Skikne BS, Cook JD. Serum transferrin receptor is a truncated form of tissue receptor. J Biol Chem **265**:19077–19081, 1990.
11. Shih YJ, Baynes RD, Hudson BG, Cook JD. Characterization and quantitation of the circulating forms of serum transferrin receptor using domain specific antibodies. Blood **81**:234–238, 1993.
12. Baynes RD, Shih YJ, Hudson BG, Cook JD. Characterization of transferrin receptor released by K562 erythroleukemia cells. Proc Soc Exp Biol Med **197**:416–423, 1991.
13. Baynes RD, Shih YJ, Cook JD. Production of soluble transferrin receptor by K562 erythroleukemia cells. Br J Haematol **78**:450–455, 1991.
14. Baynes RD, Shih YJ, Hudson BG, Cook JD. Identification of the membrane remnants of transferrin receptor using domain specific antibodies. J Lab Clin Med **123**:407–414, 1994.
15. Baynes RD, Skikne BS, Cook JD. Circulating transferrin receptors and assessment of iron status. J Nutr Biochem **5**:322–330, 1994.
16. Baynes RD, Shih YJ, Hudson BG, Cook JD. Production of the serum form of the transferrin receptor by a cell membrane associated serine protease. Proc Soc Exp Biol Med **204**:65–69, 1993.
17. Klausner RD, Rouault TA, Harford JB. Regulating the fate of mRNA: The control of cellular iron metabolism. Cell **72**:19–28, 1993.
18. Kohgo Y, Niitsu Y, Nishisato T, Kato J, Sasaki K, Tsushima N, Hirayama M, Kondo H, Urushizaki I. Externalization of transferrin receptor in established human cell lines. Cell Biol Int Rep **11**:871–879, 1987.
19. Chitambar CR, Zivkovic-Gilgenbach Z. Influence of cellular iron status on the release of soluble transferrin receptor from

human promyelocytic leukemic HL60 cells. J Lab Clin Med **116**:345–353, 1990.
20. Edwards J, Huebers H, Kunzler C, Finch C. Iron metabolism in the Belgrade rat. Blood **67**:623–628, 1986.
21. El Guindi M, Skikne BS, Covell AM, Cook JD. An immunoassay for human transferrin. Am J Clin Nutr **47**:37–41, 1988.
22. Klausner RD, Harford J, Van Renswoude J. Rapid internalization of the transferrin receptor in K562 cells is triggered by ligand binding or treatment with a phorbol ester. Proc Natl Acad Sci USA **81**:3005–3009, 1984.
23. Chitambar CR, Zivkovic Z. Release of soluble transferrin receptor from the surface of human leukemic HL60 cells. Blood **74**:602–608, 1989.
24. Girones N, Davis RJ. Comparison of the kinetics of cycling of the transferrin receptor in the presence or absence of bound diferric transferrin. Biochem J **264**:35–46, 1989.