Hemin regulates the expression of transferrin receptors in human hematopoietic cell lines

P.G. Pelicci*, A. Tabilio*, P. Thomopoulos+, M. Titeux†, W. Vainchenker†, H. Rochant† and U. Testa†

† *Unité de Recherches sur les Anémies, INSERM U.91, Hôpital Henri Mondor, 94010 Creteil,* and + *Unité de Recherches sur le Métabolisme des Lipides INSERM U.35, Hôpital Henri Mondor, 94010 Creteil, France,* and * *Istituto di Clinica Medica Generale e Terapia Medica Università di Perugia, Perugia, Italy*

Received 17 June 1982; revision received 9 July 1982

Iron uptake *Transferrin receptors* *Hemin*

1. INTRODUCTION

It was noted that all cultured cells require transferrin for optimal growth in a serum-free medium [1]. In addition there is evidence indicating the existence of transferrin receptors in almost all the human cell lines grown in vitro [2–13].

Heme is known to play an important role in mammalian cells: (1) it is a structural component of hemoglobin; (2) it regulates the expression of messenger RNA at the translational level [14]; (3) it affects the rate of the transcription of the globin mRNA [15]; and (4) it decreases the proteolysis of abnormal proteins in erythroid cells [16]. In addition, previous studies have shown that hemin regulates iron incorporation into protoporphyrin [17]. More particularly, the addition of hemin to reticulocytes reduces the iron uptake [18], while the incubation of reticulocytes with heme inhibitors increases the Fe uptake [19].

This prompted us to investigate the effect of the hemin on the expression of transferrin receptors in several human hematopoietic cell lines. The results have shown that hemin constantly decreased the binding of transferrin to the cells grown in vitro without affecting the affinity of transferrin for its receptor.

Address correspondence to: Dr. Ugo Testa, INSERM U.91, Hôpital Henri Mondor, 94010. Creteil, France

2. MATERIALS AND METHODS

2.1. *Culture*

Four different human hematopoietic cell lines were studied: K 562 [20], HL-60 [21], Raji [22] and CCRF-CEM [23]. The conditions of culture were previously reported [24]. To investigate the effect of hemin, K 562 was incubated for 4 days in the presence of 100 μM hemin (Sigma, USA); HL-60, Raji and CCRF-CEM were grown in the presence of 50 μM hemin in order to not affect cell proliferation or viability. Under these conditions of culture, hemin did not affect the growth rate of the cell lines.

2.2. *Radiolabelling of transferrin*

Purified human transferrin was obtained from Sigma (USA). It was conjugated with ^{125}I by the solid phase lactoperoxidase method (New England, Radio iodination system).

2.3. *Radio-iodinated transferrin binding assays*

The binding conditions were performed in the conditions previously reported [24]. Briefly, 1×10^6 cells were incubated for 30 min at 20°C in the presence of a constant amount of ^{125}I-Trf (50 ng) and in the presence of increasing amounts of unlabelled transferrin (from 50 ng to 10 μg).

2.4. *Immunofluorescence labelling*

The cells were washed 3 times with Hanks' saline solution and incubated for 30 min in a 1:500 or

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

1:1000 concentration of B3/25 monoclonal antibody [25,26]. After three additional washes, the cells were incubated with $F(ab')_2$ rabbit anti-mouse IgG conjugated with fluorescein for 30 min. The cells were then examined under a Zeiss microscope equipped for fluorescence and the percentage of fluorescent cells was determined.

2.5. *Gel filtration*

K 562 cells were dissolved in PBS 10 mM (pH 7.40) containing 1% Triton X-100, phenylmethansulphonylfluoride (PMSF) at 1 mM. After incubation at 4°C for 15 min, the sample was centrifuged for 30 min at 13 000 × g. The supernatant was mixed with a saturating amount of ^{125}I-Transferrin, incubated at 37°C for 15 min and then chromatographed on a column (1 × 40 cm) of Sephadex G-200 (Superfine, Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M Tris–citrate (pH 5.0) containing Triton X-100 0.1%.

2.6. *Solubilized transferrin receptor assay*

A simple assay was devised to measure solubilized transferrin receptor activity. It is based on a difference in solubility of free and transferrin bound receptors in polyethylene glycol (PEG). Dissolved receptors (Triton X-100 1%) were incubated in a total volume of 0.2 ml for 30 min at 37°C in a 0.1 M citrate–Tris buffer solution (pH 5.0) containing 0.1% bovine serum albumin (BSA), 0.1% Triton X-100 and 5 ng of ^{125}I-transferrin. The receptor transferrin complex was precipitated with 0.8 ml of PEG solution (12% w/v) in 0.1 M citrate–Tris buffer (pH 5.0) containing BSA 0.1%, Triton X-100 0.1% and the carrier human gammaglobulin 0.1%. The tubes were placed in an ice-bath for 30 min and then centrifuged at 13 000 × g for 15 min at 4°C. The supernatant and the precipitate were tested for radioactivity. Co-precipitation of free transferrin was measured by omitting the receptor from the tubes while non specific binding of transferrin was determined by pre-incubating the samples with 1 mg of non-radioactive transferrin before adding the radioactive transferrin.

3. RESULTS

3.1. *Transferrin binding to four human cell lines after hemin induction*

The results of the binding of ^{125}I-transferrin to four hematopoietic cell lines have constantly shown that 4 days after hemin addition to the cultures, transferrin binding was markedly reduced with respect to the control (table 1). The decrease of ^{125}I-Trf binding induced by hemin was dose dependent (table 2). At all the concentrations tested hemin did not affect the rate of growth of the cells. In order to investigate the time required for hemin to affect the transferrin binding, we incubated the cells with hemin and then we investigated transferrin binding at various times. Transferrin binding

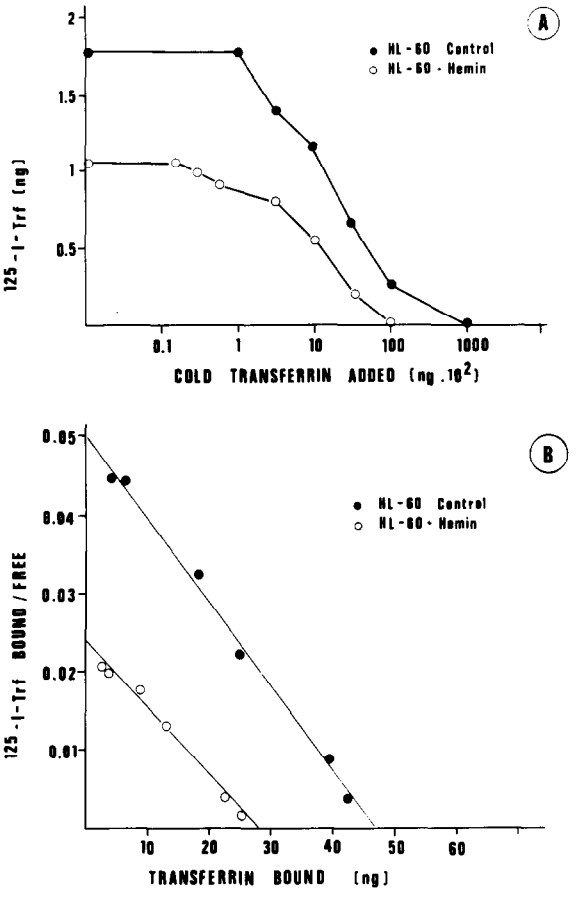

Fig.1. Scatchard plot of bound transferrin against the ratio of bound to free lingand cells reacted in competitive binding assays with a constant amount of ^{125}I-transferrin (50 ng) and increasing amounts of ligand (2 ng–10 μg). The average number of receptor sites was estimated from the X intercept.

Table 1

Effect of hemin on ^{125}I-Trf binding in human hematopoietic cell lines

	Control ^{125}I-Trf bound (ng/10^{6}cells)	K_{aff} (M)	Hemin ^{125}I-Trf bound (ng/10^6 cells)	K_{aff} (M)
K 562	17	1.3×10^{-12}	4	1.4×10^{-12}
Hl 60	20	1.5×10^{-12}	8	1.4×10^{-12}
Raji	3.5	1.5×10^{-12}	2	1.7×10^{-12}
CCRF-CEM	9	1.3×10^{-12}	4	1.7×10^{-12}

The difference between the ng of Trf bound in the control and in samples treated with hemin was significant ($t = 4.89$, $P > 0.005$); in contrast, the constant of affinity was not significantly different in the control and in TPA treated cells ($t = 0.83$, $P > 0.4$)

began to decrease as early as 10 hours after incubation in the presence of hemin (table 3).

The affinity of receptors was examined by means of competitive binding experiments and the results were expressed in the form of Scatchard plots [27]. The results concerning HL-60 cell line are shown in the fig.1.

The affinity of binding (K_{aff}) was not significantly modified by hemin induction (table 1). In contrast, the number of transferrin receptors significantly decreased after hemin induction (table 1). The addition of hemin to the binding mixture did not directly affect the binding of transferrin to the cells (data not shown).

The effect of hemin on the binding of transferrin can not be dependent on a decreased rate of cell growth since hemin did not inhibit the cellular proliferation and the incorporation of thymidine as we have previously shown [28,29].

3.2. *Expression of transferrin receptor defined by immunofluorescence labelling using a B3/25 monoclonal antibody*

The cellular expression of the transferrin recep-

Table 2

Effect of increasing concentration of hemin on transferrin binding

Hemin (μM)	^{125}I-Transferrin bound (% of control)
0	100
5	90
10	85
25	70
50	45

HL-60 cells were grown for three days in the presence of increasing concentrations of hemin and ^{125}I-Trf binding was then investigated

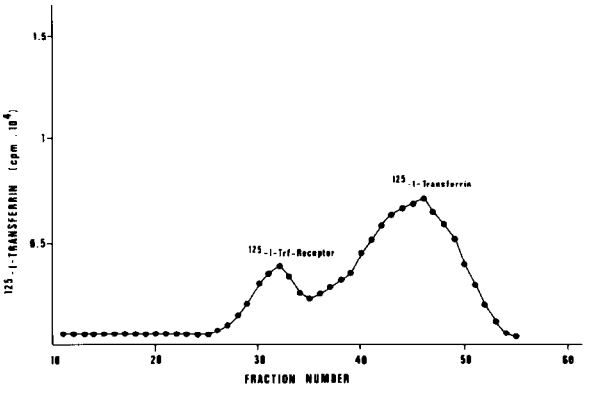

Fig.2. Gel filtration on G-200 Sephadex of a solubilized cellular sample (K 562 cells) preincubated in the presence of an excess of ^{125}I-Trf.

Table 3

Kinetics of the effect of hemin on the binding of ^{125}I-transferrin

Time (h) after hemin addition	^{125}I-Trf binding (% of the control)
0	100
1	105
2	102
3	98
10	78
20	75
40	60

50 μM hemin was added to 50 ml of 2 × 10^5/ml HL-60 cells and the ^{125}I-transferrin binding was investigated at different times after hemin addition

Table 4

Investigation of transferrin receptor by immunofluorescence

Cell line	Addition to the medium	% Labelled cells
HL 60	None	100
HL 60	Hemin, 0.1 mM	49
K 562	None	88
K 562	Hemin, 0.1 mM	63

Transferrin receptor expression was investigated by immunofluorescence labelling using B3/25 monoclonal antibody. The difference between the number of cells labelled by B3/25 antibody in the control and in hemin treated cells was significant ($t = 3.5$, $P > 0.1$)

Table 5

Investigation of solubilized transferrin receptors expression by PEG assay

	K 562		HL 60	
	Control	Hemin	Control	Hemin
^{125}I-Trf binding (μg/mg protein)	0.3	0.17	0.39	0.19

The difference in ^{125}I-transferrin binding between the control and hemin treated cells was significant ($t = 4.7$, $P > 0.05$)

tor was investigated by immunofluorescence using B3/25, a monoclonal antibody which specifically recognizes the receptor for transferrin [31,32]. Hemin decreased the proportion of the cells labelled by this antibody (table 4).

3.3. *Transferrin receptor assay on solubilized membranes*

The above results could be the consequence either of a true decrease of transferrin receptors or of their exposure to the membrane. In consequence, we have measured the transferrin receptor on dissolved membrane samples. In order to perform this assay we have complexed ^{125}I-transferrin with cell samples dissolved in Triton X-100 (1%) and then precipitated the transferrin receptor complex in 12% PEG solution. Under our experimental conditions, transferrin forms a complex with its receptor as shown by gel filtration experiments (fig.2).

The PEG assay has shown that cells grown in the presence of hemin exhibit a lower amount of transferrin receptor with respect to those grown without hemin (table 3).

4. DISCUSSION

Previous experiments [24] have shown that hemin regulates the expression of the transferrin receptor on the K 562 leukemic cell line. Notably, the addition of hemin to the culture medium resulted in a reduction of receptors for transferrin on the surface of K 562 cells. Our experiments extend this observation to other human, non-erythroid, hematopoietic cell lines.

Similarly to K 562, after hemin addition, there was a significant and constant reduction of the number of transferrin binding sites per cell without any modification of the affinity of binding, examined by means of binding competition experiments. The action of hemin on the binding of transferrin to the cells was dose dependent. Furthermore, hemin induced a reduction of the proportion of cells exhibiting the transferrin receptor as investigated by immunofluorescence labelling with B3/25 monoclonal antibody [25,26].

In order to rule out that the action of hemin merely consists in modifying the accessibility of the transferrin binding sites on the membrane, without reducing their real number, we measured

the transferrin binding on solubilized membrane samples. In these experiments hemin elicited a reduction of transferrin binding sites comparable to that observed incubating intact cells with ^{125}I-transferrin. These results afford evidence that hemin acts by reducing the real number of transferrin receptors present on the cell surface.

Thus, the proliferation status [30–32] and hemin are the only known factors that regulate the expression of transferrin receptors. Furthermore, previous studies have shown that brief incubations in the presence of hemin inhibit iron uptake by reticulocytes [18,19] and that this phenomenon could be related to an inhibition of iron release from transferrin and not to a reduction in transferrin binding to the cells [18,19]. Our findings may therefore indicate the presence of another mechanism by which heme regulates the uptake of iron by the cells, i.e. via the number of transferrin receptors.

ACKNOWLEDGEMENTS

We are extremely grateful to Dr. Trowbridge for this generous gift of the B3/25 monoclonal antibody. We extend our thanks also to A.M. Dulac and M. Segear for typing the manuscript. This work was supported by a grant from INSERM.

REFERENCES

[1] Barnes, D. and Sato, G.H. (1980) Cell 22, 649.
[2] Philips, G.L. (1976) Biochem. Biophys. Res. Commun. 72, 634.
[3] Galbraith, G.M.P., Goust, J.M., Mercurio, S.M. and Galbraith, R.M. (1980) Clin. Immunopathol. 16, 387.
[4] Galbraith, R.M., Werner, P., Arnaud, P. and Galbraith, G.M.P. (1980) J. Clin. Invest. 66, 1135.
[5] Hu, H.Y.Y., Gardner, J., Aisen, P. and Skoultchi, A.I. (1977) Science 197, 599.
[6] Fernandez-Pol, J.A. and Klos, D.J. (1980) Biochemistry 19, 3904.
[7] Faulk, W.P., Hsi, B.L. and Stevens, P.J. (1980) Lancet ii, 390.
[8] Octave, J.N., Schneider, Y.J., Crichton, R.R. and Trouet, A. (1981) Eur. J. Biochem. 115, 611.
[9] Young, S.P. and Aisen, P. (1980) Biochim. Biophys. Acta 633, 145.
[10] Hamilton, T.A., Wada, H.G. and Stssman, H.H. (1979) Proc. Natl. Acad. Sci. USA 76, 6406.
[11] Larrick, J.W. and Cresswell, P. (1979) Biochim. Biophys. Acta 583, 48.
[12] Galbraith, G.M.P., Galbraith, R.M. and Faulk, W.P. (1980) Cell. Immunol. 49, 215.
[13] Salomon, D. (1980) Exp. Cell. Res. 128, 311.
[14] Ross, J. and Sautner, D. (1976) Cell 8, 513.
[15] London, I.M., Cleureus, M.J., Ranu, R.S., Levin, D.A., Cherbes, L.F. and Ernst, V. (1976) 35, 2218.
[16] Etlinger, J.D. and Poldberg, A.L. (1980) J. Biol. Chem. 255, 4563.
[17] Malik, Z., Halbrecht, I. and Djaldetti, M. (1979) Differentiation 13, 77.
[18] Ponka, P., Neuwirt, J. and Borove, J. (1976) Enzyme 17, 91.
[19] Ponka, P., Wilczinska, A. and Schulman, H.M. (1982) Biochim. Biophys. Acta 720, 96.
[20] Lozzio, C.B. and Lozzio, B.B. (1975) Blood, 45, 321.
[21] Collins, S.T., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C. (1978) Proc. Natl. Acad. Sci. USA 75, 1458.
[22] Pulvertaft, R.J.V. (1969) J. Clin. Pathol. 18, 261.
[23] Foley, G.E., Lazarus, H., Farber, S., Uzman, B., Boone, V.A. and Mc Carthy, R.E. (1965) Cancer 18, 522.
[24] Testa, U., Thomopoulos, P., Vinci, G., Titeux, M., Bettaieb, A., Vainchenker, W. and Rochant, H. (1982) Exp. Cell. Res. in press.
[25] Trowbridge, I.S. (1978) J. Exp. Med. 148, 313.
[26] Omary, M.B., Trowbridge, I.S. and Minowada, J. (1980) Nature 286.
[27] Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51. 660.
[28] Guerrasio A., Vainchenker, W., Breton-Gorius, J., Testa, U., Rosa, J., Thomopoulos, P., Titeux, M., Guichard, J. and Beuzard, Y. (1981) Blood Cells 7, 165.
[29] Vainchenker, W., Testa, U., Guichard, J., Titeux, M. and Breton-Gorius, J. (1981) Blood Cells 7, 357.
[30] Sutherland, R., Delia, D., Schneider, C., Newman, R., Kemshead, J. and Greaves (1981) Proc. Natl. Acad. Sci. USA 78, 4515.
[31] Trowbridge, I.S. and Omary, M.B. (1981) Proc. Acad. Natl. Sci. USA 78, 3039.
[32] Judd, W., Poodry, C.A. and Strominger, J.L. (1980) J. Exp. Med. 152, 1430.