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Specific binding to plasma membrane is the first step in the uptake of non-transferrin iron by cultured cells

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Abstract

We studied transport of non-transferrin iron into HeLa cells adapted for growth in defined medium, containing either 5 μg/ml of iron-saturated transferrin (HeLa/Tf cells) or 5 μM ferric citrate (HeLa/Fe5 cells) as a source of iron. Employing ^{55}Fe-ferric citrate, iron uptake by intact cells was compared with iron binding to isolated membranes. Uptake characteristics of both HeLa/Tf and HeLa/Fe5 cells seemed to be similar: K_m = 14 μM and V_{max} = 135 pmol Fe/min/10^5 cells for HeLa/Tf, K_m = 22 μM and V_{max} = 165 pmol Fe/min/10^5 cells for HeLa/Fe5. Increasing concentrations (0.3–1.2 μM) of ^{55}Fe-ferric citrate, producing levels of free ^{55}Fe which were independent of total Fe under the experimental conditions used, led to increased binding of ^{55}Fe for both HeLa/Tf and HeLa/Fe5 cells (1.08–8.03 nmol Fe/h/10^5 cells). This corresponds with the suggestion that iron was bound in the form of ferric citrate rather than in the form of free iron. Dissociation constants of Fe binding, K_D = 0.61 μM for HeLa/Tf and K_D = 1.53 μM for HeLa/Fe5, were obtained from competition experiments. We conclude that specific binding sites for ferric citrate are constitutively expressed in plasma membrane and that their expression does not require the induction by the presence of ferric citrate. The uptake of non-transferrin iron is realized in at least two steps. The first step is iron binding to the specific binding sites in plasma membrane. The binding does not represent a limiting step of the uptake. © 1998 Elsevier Science B.V.

Keywords: Non-transferrin iron transport; Iron uptake; Iron binding site; HeLa cell

1. Introduction

Iron is transported by the serum glycoprotein transferrin in vertebrates. Animal cells acquire transferrin-bound iron by receptor-mediated endocytosis of transferrin–transferrin receptor complex [1,2]. Transferrin-dependent iron transport is not the only pathway of iron uptake. Previous studies have documented transferrin-independent iron uptake by cells both, in vivo [3–6] and in vitro [7–13]. HeLa cells were shown to take up ferric as well as ferrous iron from complexes with citrate, ascorbate and nitrilotriacetate [10,14,15]. However, the mechanism of non-

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transferrin iron transport has not been elucidated until now.

There are several reasons to suppose that the process of non-transferrin iron uptake is of physiological significance. One of the reasons is connected with the possibility that tumor cells utilize non-transferrin iron uptake as an alternative pathway for iron acquisition [7,13]. In normal human plasma, only a small portion (ca. 1%) of total iron is bound to low-molecular-weight complexes, mainly to citrate [16]. However, significantly higher local concentrations of non-transferrin iron caused by cell necroses and release of cytoplasmatic pools of iron are likely in the tumor microenvironment [8,17]. Moreover, the non-transferrin iron transport across the cell membrane could be analogous to the passage of iron from endocytic vesicles after its release from internalized transferrin [11]. It could also tell us something about internalization of other metals [10].

The present study deals with non-transferrin iron transport into HeLa cells. Iron uptake by the cells was compared with iron binding to isolated cell membranes. We found that there are specific binding sites for iron in plasma membrane. These binding sites are constitutively expressed. Binding to these sites is the first though not limiting step of non-transferrin iron uptake.

2. Materials and methods

2.1. Cells and culture conditions

A clone of the human cell line HeLa (cervical carcinoma), growing as a suspension culture, was obtained from A. Cvekl (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic). HeLa/Tf cells and HeLa/Fe5 cells are cells adapted for long-term growth in Tf medium and Fe5 medium, respectively. Tf medium comprises PFH medium, described previously [18], with 5 μg/ml of iron-saturated human transferrin instead of 500 μM ferric citrate. Fe5 medium comprises PFH medium, containing only 5 μM ferric citrate [19,20]. PFH medium is based on RPMI 1640 medium. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. Iron uptake measurement

We employed a method derived from the method used by Inman and Wessling-Resnick [11]. Cells were harvested by low-speed centrifugation (10 min at 500 × *g*) and washed twice with the medium lacking transferrin or ferric citrate and used immediately. The incubation performed at 37°C (4°C), in a final volume of 0.5 ml, was started by the addition of cells ($0.2–0.3 \times 10^6$ cells) to the incubation medium. The incubation medium contained ferric citrate (final Fe concentration of 0.6–300 μM including 0.6 μM ^{55}Fe-ferric citrate as a tracer) at Fe : citrate molar ratio of 1 : 1.5. After the incubation period, the uptake was stopped immediately by rapid vacuum filtration of the incubation mixture on Whatman GFC fibre glass filters. Filters were washed thrice with 5 ml of ice-cold PBS. Radioactivity of the cells retained on the filters was measured by liquid scintillation in a Beckman LS 7800 counter.

Values for iron uptake in the initial part of time dependence were supposed to be influenced by iron binding to the membrane sites. However, the saturation of the binding was found to be very rapid (1.5–3 min) at 37°C. Therefore, the rate of the uptake, which was calculated from the approximately linear part of time-dependence curves (1.5–17 min) was not influenced significantly by the initial iron binding. Radioactivity associated with the cells after the incubation at 4°C, which was mainly related to the non-specific binding, represented only ca. 10–20% of the radioactivity associated with the cells after the incubation at 37°C for individual experiments.

2.3. Preparation of membrane fraction

Cells were harvested by low-speed centrifugation (10 min at 500 × *g*) and washed twice with the medium lacking transferrin or ferric citrate. Washed cells were resuspended in ice-cold PBS and homogenized with a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged for 10 min at 700 × *g*. Supernatant was collected and centrifuged for 30 min at 60 000 × *g*. Supernatant was removed and the pellet was resuspended in ice-cold 25 mM HEPES buffer (pH 7.4) at a concentration corresponding to $10–20 \times 10^6$ cells/ml. Membranes were stored at −20°C no longer than 2 months.

2.4. Measurement of iron binding to cell membranes

The binding of ^{55}Fe to membrane fractions of Hela/Tf and Hela/Fe5 cells incubated in increasing concentrations of ^{55}Fe-ferric citrate (0.3–1.2 μM) was measured as follows: the incubation was performed at 25°C in a final volume of 0.5 ml. Membrane fraction corresponding to 2×10^6 cells was added to the incubation buffer (25 mM HEPES, pH 7.4) containing the required amount of ^{55}Fe-ferric citrate and incubated for 1 h. The competition between ^{55}Fe binding from ^{55}Fe-ferric citrate and cold Fe binding from increasing concentrations of ferric citrate in equilibrium was measured as follows: the incubation was again performed at 25°C in a final volume of 0.5 ml. Membrane fraction corresponding to 0.5×10^6 cells was added to the incubation buffer (25 mM HEPES, pH 7.4) containing ^{55}Fe-ferric citrate (final concentration of 0.6 μM) plus the required amount of cold ferric citrate (10^{-9}–10^{-4} M) and incubated for 30 min. The incubations were stopped by rapid vacuum filtration of the incubation mixtures on Whatman GFC fiber glass filters. Filters were washed thrice with 5 ml of ice-cold 5 mM HEPES. Radioactivity of membranes retained on filters was measured by a Beckman LS 7800 liquid scintillation counter.

Acquired competition data were analyzed by nonlinear regression employing the program ENZFITTER and using the equation $B/B_{max} = L^{nH}/(IC_{50}^{nH} + L^{nH})$ [21,22], where n_H is the Hill slope factor, B/B_{max} the fraction of occupied binding sites and L the concentration of radioligand (constant during the experiment).

3. Results

3.1. Iron uptake by cells

We studied iron accumulation from ferric citrate-containing medium by HeLa/Tf cells (HeLa cells adapted for long-term growth in transferrin medium containing 5 μg/ml of transferrin as a source of iron) and by HeLa/Fe5 cells (HeLa cells adapted for long-term growth in Fe5 medium containing 5 μM ferric citrate as a source of iron).

The accumulation from 0.6–300 μM ferric citrate increased more or less linearly during the first 1.5–17 min for both HeLa/Tf and HeLa/Fe5 cells (Figs. 1 and 2). After 17 min, the values of accumulated iron by HeLa/Tf cells were in the 0.038–4.600 nmol

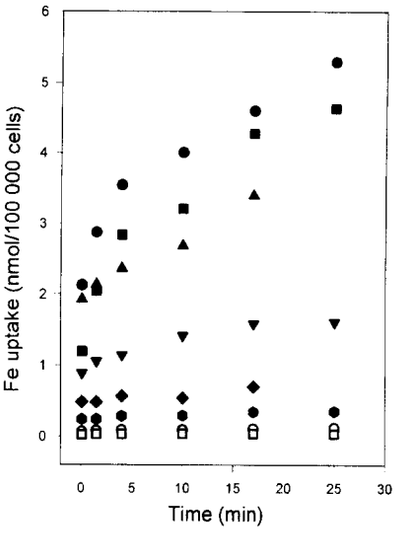

Fig. 1. Time dependence of iron uptake by HeLa/Tf cells at 37°C incubated in various concentrations of ferric citrate: (●) – 100 μM, (■) – 30 μM, (▲) – 18 μM, (▼) – 10 μM, (◆) – 5.8 μM, (filled hexagon) – 3.4 μM, (○) – 1.6 μM and (□) – 0.6 μM. All ferric citrate concentrations contained 0.6 μM ^{55}Fe as a tracer. Each point represents the mean of two independent experiments.

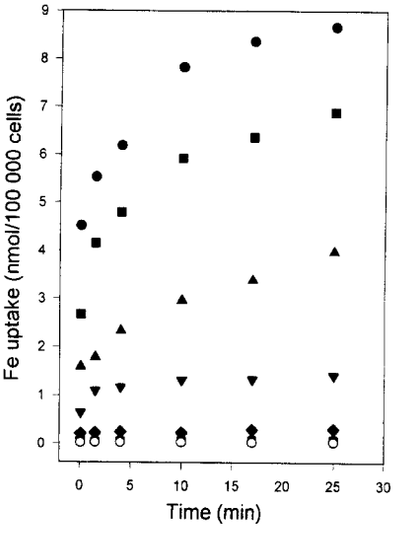

Fig. 2. Time dependence of iron uptake by HeLa/Fe5 cells at 37°C incubated in various concentrations of ferric citrate: (●) – 300 μM, (■) – 100 μM, (▲) – 30 μM, (▼) – 10 μM, (◆) – 3.4 μM, (filled hexagon) – 1.6 μM and (○) – 0.6 μM. All ferric citrate concentrations contained 0.6 μM ^{55}Fe as a tracer. Each point represents the mean of two independent experiments.

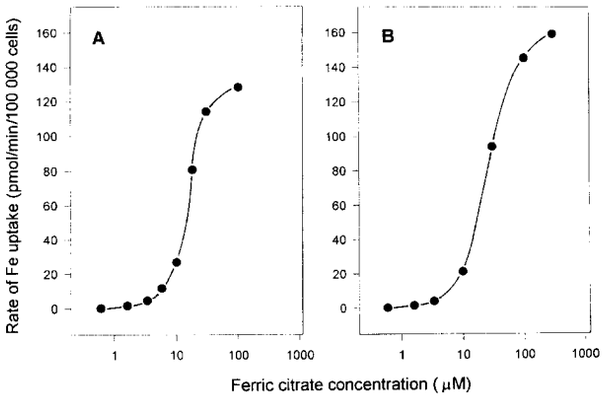

Fig. 3. The effect of ferric citrate concentration on the rate of Fe uptake by (A) HeLa/Tf and (B) HeLa/Fe5 cells. ^{55}Fe-ferric citrate (0.6 M) was used as a tracer. Rate values were calculated by linear regression from the linear part of time-dependence curves of iron uptake (see Figs. 1 and 2).

Fe/10^5 cells range for extracellular 0.6–100 μM ferric citrate. HeLa/Fe5 displayed values 0.029–8.368 nmol Fe/10^5 cells for 0.6–300 μM ferric citrate.

Uptake values in the approximately linear part of the time-dependence curves (1.5–17 min) were used to calculate the rate of iron uptake by a linear regression. The rate of the uptake was plotted against ferric citrate concentration (Fig. 3(A) and (B)). We assessed K_m and V_{max} from the curves to be K_m = 14 μM and V_{max} = 135 pmol Fe/min/10^5 cells for HeLa/Tf and K_m = 22 μM and V_{max} = 165 pmol Fe/min/10^5 cells for HeLa/Fe5 cells. Uptake characteristics of HeLa/Tf and HeLa/Fe5 seemed to be similar.

Table 1
^{55}Fe binding to membrane fractions of HeLa/Tf and HeLa/Fe5 cells incubated in various concentrations of ^{55}Fe-ferric citrate

Cell type	^{55}Fe concentration (μM)	Bound ^{55}Fe [a] (nmol/10^5 cells)
HeLa/Tf	0.3	1.08
	0.6	2.90
	1.2	5.78
HeLa/Fe5	0.6	3.74
	1.2	8.03

[a] After 1 h incubation at 25°C. Data are from one representative experiment.

3.2. Iron binding to plasma membrane

Our preliminary experiments showed that Fe binding to membrane fractions of HeLa/Tf as well as of HeLa/Fe5 cells is a relatively rapid proces. The equilibrium at 25°C was reached within 10–15 min. At 4°C, the reaction was in equilibrium within 1 h.

^{55}Fe binding to the membranes increased with increasing concentration of ^{55}Fe-ferric citrate for both HeLa/Tf and HeLa/Fe5 cells (Table 1). This corresponds with the suggestion that iron binds in the form of ferric citrate rather than in the form of free iron when considering the extremely high constant of complex stability of $10^{11.5}$ M^{-1} [23] and the 1.5-fold excess of citrate in the reaction (see Section 4). The binding to HeLa/Fe5 cells seems to be somewhat higher than the binding to HeLa/Tf.

When membranes were incubated with 0.6 μM ^{55}Fe-ferric citrate plus increasing concentrations of cold ferric citrate, the ^{55}Fe binding decreased and reached total inhibition at 10^4 M cold ferric citrate (Fig. 4(A) and (B)). The curves fitted best the model of one type of binding site. The Hill coefficient n_H was found to be close to unity (1.01 for HeLa/Tf and 1.09 for HeLa/Fe5). This points strongly to the presence of only a single class of binding sites, or to the possibility that the contribution of other binding sites is not significant. The dissociation constant of Fe binding calculated from the IC_{50} was K_D = 0.61 μM for HeLa/Tf cells and K_D = 1.53 μM for HeLa/Fe5 cells. K_D for both, HeLa/Tf and

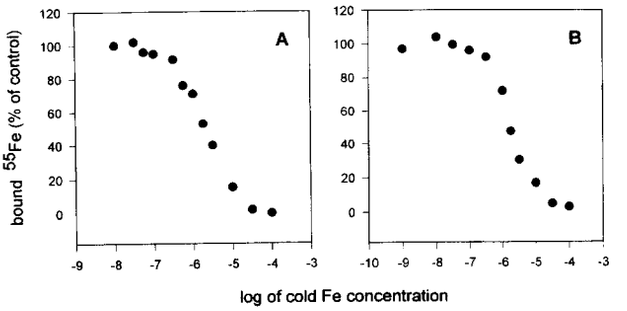

Fig. 4. Binding of ^{55}Fe from ^{55}Fe-ferric citrate (0.6 μM) to membrane fractions of (A) HeLa/Tf and (B) HeLa/Fe5 cells in the presence of increasing concentrations (10^{-9}–10^{-4} M) of cold Fe (ferric citrate) at 25°C. Control represents ^{55}Fe binding without the presence of cold Fe. Each point is the mean of two independent experiments representing 2–3 experimental values.

HeLa/Fe5 was in the range of concentrations corresponding to the specific binding.

4. Discussion

Simple equilibrium of ferric ions forming 1 : 1 complex with citrate is given by a stability constant which is $\approx 10^{11.5}\,M^{-1}$ [23]. Non-transferrin iron used in our study was in the form of ferric citrate with the molar ratio Fe : citrate of 1 : 1.5. In the presence of 1.5-fold excess citrate, most of the ferric iron is bound as the citrate complex. Only ca. 10^{-11} M iron is present as free iron independently of the total iron concentration within the 10^{-3}–10^{-7} M range (Fig. 5). This means that a change of total ferric citrate concentration within the foregoing range does not affect the apparent concentration of free iron. If iron binds to the membrane as free iron, an increase in ferric citrate concentration within the mentioned range would not lead to increased iron binding. However, in our experiments increased levels of ^{55}Fe-ferric citrate resulted in increased binding of ^{55}Fe (see Table 1). Therefore, we can assume that iron binds to the membrane in the form of ferric citrate and, thus, we can assess K_D of the binding by a competition experiment with cold ferric citrate.

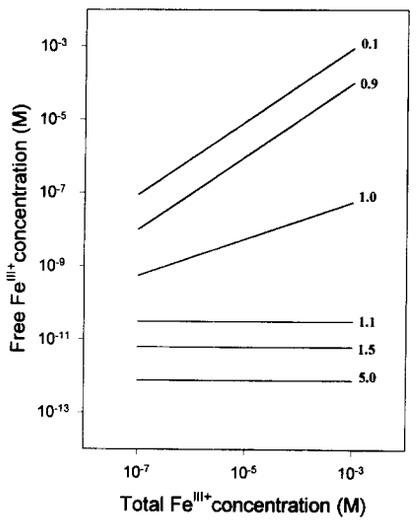

Fig. 5. Dependence of free ferric iron concentration on total ferric citrate concentration for various molar ratios Fe : citrate (1 : 0.1, 0.9, 1.0, 1.1, 1.5, 5.0). Data were obtained by theoretical calculations using an equation derived from the formula for the stability constant of the ferric citrate complex.

A competition experiment with isolated membranes of HeLa/Tf (cells grown in a medium with 5 μg/ml of transferrin) and HeLa/Fe5 (cells grown in a medium with 5 μM of ferric citrate) showed total inhibition of ^{55}Fe binding by increasing concentrations of cold ferric citrate (see Fig. 4). K_D values correspond with a specific binding and the slope of the competition curve corresponds with binding to one type of binding site. For both HeLa/Tf and HeLa/Fe5 cells K_D values are comparable (0.61 μM and 1.53 μM). This means that the binding sites for ferric citrate are, in general, constitutively expressed in plasma membrane and their expression does not require induction by the presence of ferric citrate. Such a suggestion is also supported by similar K_m values (14 μM and 22 μM) for both HeLa/Tf and HeLa/Fe5.

However, slightly increased Fe binding to HeLa/Fe5 membranes than to HeLa/Tf membranes (Table 1) seems to be reproducible. Such an increase could represent a certain degree of upregulation of the binding sites due to previous cultivation of HeLa/Fe5 cells in the presence of non-transferrin iron.

If we compare K_D and K_m values for both HeLa/Tf and HeLa/Fe5 cells, K_m values are roughly one order of magnitude higher than K_D values. This means that specific non-transferrin iron uptake is carried out in at least two steps. The first step is iron binding to specific binding sites in plasma membrane. The binding does not represent a limiting step of the uptake.

Melanotransferrin, known as p97 and structurally similar to serum transferrin [24], represents an obvious candidate for a specific iron binding site in the plasma membrane of mammalian cells. Iron uptake by our HeLa cells represents a process with relatively low affinity (K_m ca. 20 μM) and high capacity (V_{max} ca. 1500 pmol/min/10^6 cells). Kennard et al. [25] found rather differing values (K_m ca. 2 μM, a capacity of ca. 1 pmol/min/10^6 cells) for p97 in CHO cells representing a process with higher affinity and much lower capacity. Our recent data shows that p97 is expressed on HeLa cells. Treatment of the cells with phosphatidylinositol-specific phospholipase C results in a dramatic decrease in cell surface p97 which is not, however, accompanied with a relevant decrease of iron uptake (Kriegerbecková and Kovář,

manuscript under preparation). Therefore, it seems that p97 is not involved in non-transferrin iron uptake by HeLa cells.

In conclusion, we can state that specific uptake of non-transferrin iron from ferric citrate by HeLa cells is achieved in at least two steps. The first step is binding of ferric citrate to specific binding sites in plasma membranes. The binding sites are expressed constitutively and the binding to the sites is not a limiting step of the uptake. However, the mechanism of iron transport across the membrane as well as the specific binding sites involved remain unknown.

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