TRANSFERRIN PROTEIN AND IRON UPTAKE BY ISOLATED RAT ERYTHROBLASTS

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1. Introduction

In mammals, the major proportion of body iron is found as the iron porphyrin complexes, haemoglobin, myoglobin and a variety of haem-containing enzymes. Many other iron proteins are found in the tissues, including metallo-flavoproteins and other enzymes in which iron is a cofactor. Iron is also stored within the body as ferritin and haemosiderin which are specially designed for retaining iron in a soluble and bioavailable form. Mammals have the unique ability to conserve body iron but a markedly limited capacity to absorb and excrete it (review [1]). This conservative character of iron metabolism implies multiple and rapid exchanges of the metal between different body compartments. Within the body, iron exchanges between tissues are effected by a carrier protein in the plasma, transferrin, which interacts with tissue receptors. The problem still to be elucidated is to know how, after binding of transferrin to its receptor, iron moves into the cell and between functional compounds and storage depots. Considering the ways in which transferrin iron could be assimilated by cells, a number of possibilities can be envisaged. Perhaps the most evident is that once bound to its plasma membrane receptor, the transferrin molecule would release its iron at the plasma membrane and thereafter be returned to the extracellular medium while the iron would be transported to the intracellular sites where it is required. A second possibility is that the iron-loaded transferrin molecule is internalised within the cell prior to iron release. This would be compatible with morphological evidence based on studies in reticulocytes and erythroid cells with ^{125}I-labelled transferrin and with transferrin conjugated to ferritin or to peroxidase [2,3]. On the basis of kinetic studies of the interaction of ^{59}Fe-saturated, ^{3}H-labelled transferrin with cultured rat fibroblasts, we have proposed [4] that these cells take up iron by receptor-mediated endocytosis of transferrin, release of iron inside the lysosomes as a result of the acidic pH prevailing in these granules [5] and recycling of iron-depleted transferrin to the cell surface. We have also reported [6,7] that lysosomotropic substances, in particular chloroquine and methylamine, strongly decrease the transferrin iron uptake by fibroblasts. Since these drugs increase the intralysosomal pH of macrophages from 4.8–6.2 [5], a pH at which the affinity of iron for transferrin is much higher than at pH 4.8, affect endocytosis and in particular membrane and receptor recycling [8–10]. We concluded that these data could provide us with strong arguments in favour of our working hypothesis.

An important problem is also to determine whether this mechanism is restricted to cultured fibroblasts, or whether it could be extended to other cell types. Recently, comparable results have been obtained with totipotent mouse teratocarcinoma cells and a similar model has been proposed [11].

We have therefore investigated the uptake of iron from ^{59}Fe-saturated, ^{3}H-labelled transferrin by rat erythroblasts. These red cell precursors synthesize very large amounts of haemoglobin and therefore have a requirement of iron which is many times greater than that of other cells. We report here on experiments which show that isolated rat erythroblasts display specific receptors for transferrin, at their cell surface, accumulate transferrin iron and that chloroquine and methylamine greatly reduce iron uptake from transferrin.

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2. Materials and methods

Rat serum transferrin was isolated as in [4] by chromatography on DEAE-cellulose, filtration through Ultragel 4.4 and a final chromatography on wheat germ lectin. The transferrin was labelled with ^{3}H by reductive methylation [12] using sodium [^{3}H]borohydride (5–20 Ci/mmol, Radiochemical Centre, Amersham). Apotransferrin was prepared by dialysis against 0.05 M sodium acetate buffer (pH 5.5) containing 0.05 M EDTA. The apotransferrin was labelled with ^{59}Fe as ferric citrate (25 mCi/mmol, IRE) to have a specific activity close to that of ^{3}H (~10 000 dpm/μg protein in both isotopes). The transferrin was subsequently saturated with non-radioactive iron to give an A_{465}/A_{280} ratio of 0.046 [13]. Erythroblasts were prepared according to [14]. In brief, after isolation from 16 day rat embryos (Wistar strain), the livers were homogenized in the Iscove culture medium (Gibco-Biocult, Paisley) by 5 passages through a 19 G needle. The cells obtained in these conditions were >90% erythroid cells [14]. After incubation in the presence of trypan blue, the cells were counted using a Bürker cell; on the other hand, cellular proteins were determined by the Lowry method and it was established that 1 mg protein corresponds to ~6×10^{6} cells. 2×10^{6} cells (~0.6 mg protein) were incubated at 0 or 37°C in 9 cm^{2} plastic Petri dishes or in sterile plastic tubes (Becton Dickinson, Villeneuve-la-Garenne) containing 1 ml Iscove culture medium in which the labelled transferrin had been diluted. At the end of the incubation, the cells were separated from the culture medium by centrifugation through 600 μl dibutylphthalate in propylene microtubes in a Beckmann microfuge for 30 s as in [15]. After removal of the supernatant, the cells were solubilised with 1% (w/v) sodium deoxycholate adjusted to pH 11.3 with NaOH. The radioactivity accumulated by the cells was measured using an aqualuma cocktail (Lumac Systems, Basle) in a Tri-Carb 460 CD scintillation counter (Packard Instruments, San Diego CA); all samples were corrected for quenching. Cell protein was determined by the Lowry method [16] using bovine serum albumin as a standard. The results are expressed as dpm/mg cell protein. The drugs were employed at a final concentration at which no toxic effect was detected by observation under phase contrast microscopy throughout the incubation time. For some experiments, the collected media were assayed for degradation products after precipitation of proteins by 15% (w/v) trichloroacetic acid followed by 30 min centrifugation at 1500 × *g*; the trichloroacetic acid supernatants were assayed for radioactivity as above. The nature of the cell-associated iron was analysed by isokinetic centrifugation of cell lysates on preformed linear sucrose density gradient (1.017–1.152 g/cm^{3}). After 64 h centrifugation at 4°C and 36 000 rev./min (Rotor SW41 in L550 Beckmann centrifuge), the gradient was eluted in 30 fractions and the distribution profile of the cell-associated ^{59}Fe was compared to those of labelled [^{3}H]transferrin or [^{59}Fe]haemoglobin used as standards.

3. Results

3.1. *Low temperature binding of transferrin*

At 0°C, in the presence of 10 μg/ml ^{59}Fe-loaded, ^{3}H-labelled transferrin, both isotopes attach to rat erythroblasts in comparable amounts and a plateau is reached after ~20 min (fig.1A). As a function of the [^{3}H]transferrin concentration, increasing amounts of ^{3}H label are bound to the cells but no plateau is attained (fig.1B). Assuming M_r 80 000 for transferrin and 6×10^{6} erythroblasts/mg cell protein, the Scatchard analysis of the binding parameters (fig.1C) indicates the presence of ~500 000 high affinity (K_a 2.5×10^{7} M^{-1}) sites/cell.

3.2. *Uptake of ^{59}Fe-loaded [^{3}H] transferrin*

As indicated by fig.2, after 15 min incubation at 37°C, the cells accumulate both ^{59}Fe and ^{3}H, in a process saturable with the transferrin concentration (half saturation reached at ~0.125 μM, i.e., 1/300 of the physiological concentration); the uptake of ^{59}Fe is however higher than that of ^{3}H. As a function of the incubation time (fig.3), the uptake of ^{59}Fe proceeds proportionally with time up to 6 h and then more slowly. After 20 h, it corresponds to the uptake of the iron bound to 55 μg transferrin/mg cell protein, i.e., ~20% of the amount initially present in the culture medium indicating that most probably the slow down in the uptake of ^{59}Fe results from a partial exhaustion of the medium in transferrin iron. To study the nature of the cell associated iron, homogenates of erythroblasts incubated for different times at 37°C in the presence of labelled [^{59}Fe]transferrin were analysed by isokinetic centrifugation on sucrose gradients. After 30 min incubation, half of the cell-

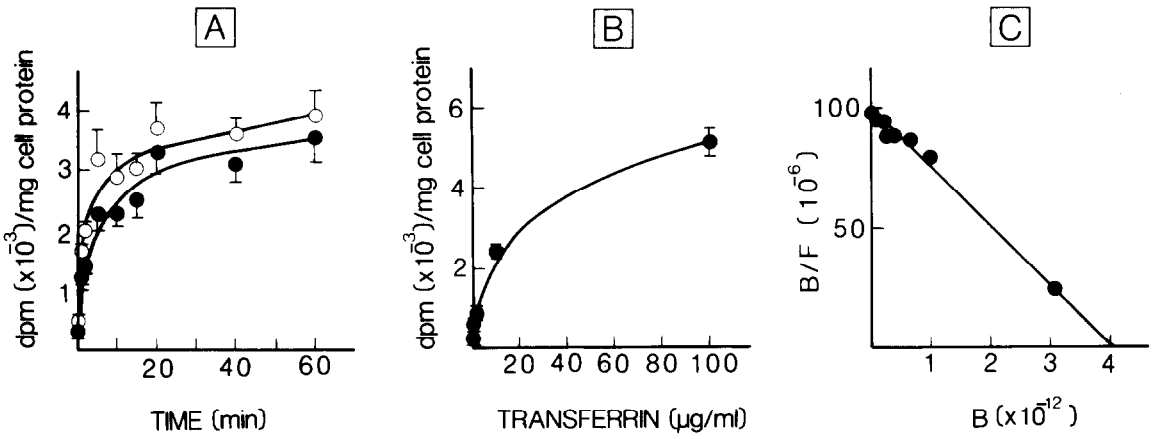

Fig.1. Erythroblasts (~300 µg protein) were incubated at 0°C for different times (A) or for 1 h (B) in 1 ml medium containing 10 µg/ml (A) or different concentrations (B) of ^{59}Fe-loaded (A) ^{3}H-labelled transferrin (A,B) (spec. act. ~10 000 dpm/µg in both isotopes). (C) Scatchard analysis of the data of (B): the abscissa represents the transferrin bound/mg cell protein; the ordinate is the ratio of bound to free transferrin: (○) ^{59}Fe; (●) ^{3}H. Mean of 3 independent experiments ± SD (A,B).

bound radioactive iron is associated with low M_r products, half with molecules behaving like ferritin. After 24 h incubation, the proportion of iron associated with ferritin does not change but ~30% of iron accompanies haemoglobin. The accumulation of ^{3}H-label by erythroblasts very rapidly reaches a plateau and after 6 h incubation the amount of cell-associated ^{3}H-label is 10-times lower than that of ^{59}Fe. In parallel to the accumulation of labelled material by the cells, no degradation products soluble in trichloroacetic acid are detected in the culture medium, even after 20 h indicating that transferrin is not digested by the cells.

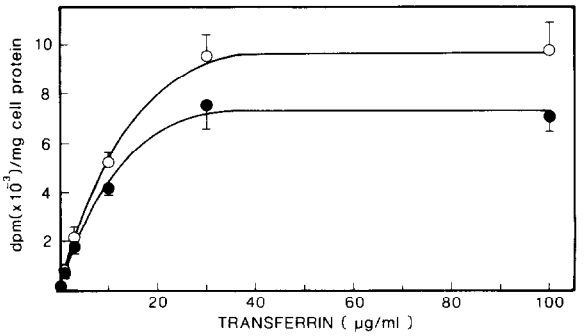

Fig.2. Cells (~300 µg protein) were incubated for 15 min at 37°C in 1 ml culture medium containing different concentrations of ^{59}Fe-loaded, ^{3}H-labelled transferrin (spec. act. as in fig.1). Mean results ± SD of 3 independent experiments are given: (○) ^{59}Fe uptake by the cells; (●) [^{3}H]transferrin uptake by the cells.

3.3. *Short term kinetic experiments*

To further investigate the mechanism of transferrin uptake, short term kinetic experiments were carried out. As shown in fig.4A, up to 2 min continuous incubation at 37°C, erythroblasts accumulate ^{59}Fe and ^{3}H in comparable amounts; after 5 min incubation, whereas the uptake of ^{59}Fe continues to increase, that of ^{3}H-label levels off and after 20 min incubation, the cells have accumulated 2.5-times more ^{59}Fe

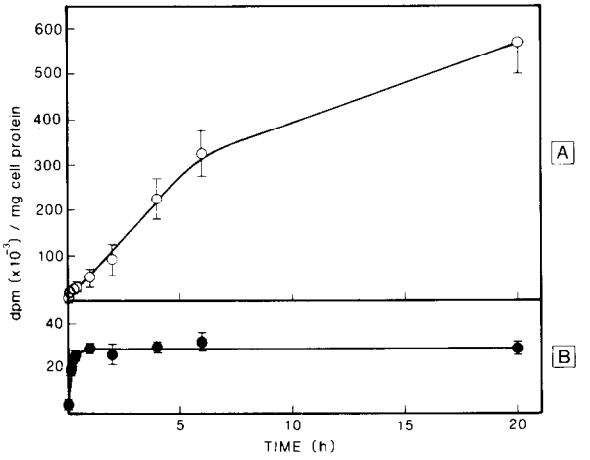

Fig.3. Cells (~300 µg protein) were incubated in 9 cm² Petri dishes for different durations at 37°C in 1 ml culture medium containing 100 µg/ml of ^{59}Fe-loaded, ^{3}H-labelled transferrin (spec. act. as in fig.1). (A) (○) ^{59}Fe uptake by the cells; (B) (●) [^{3}H]transferrin uptake by the cells. Mean results ± SD of 3 independent experiments are given.

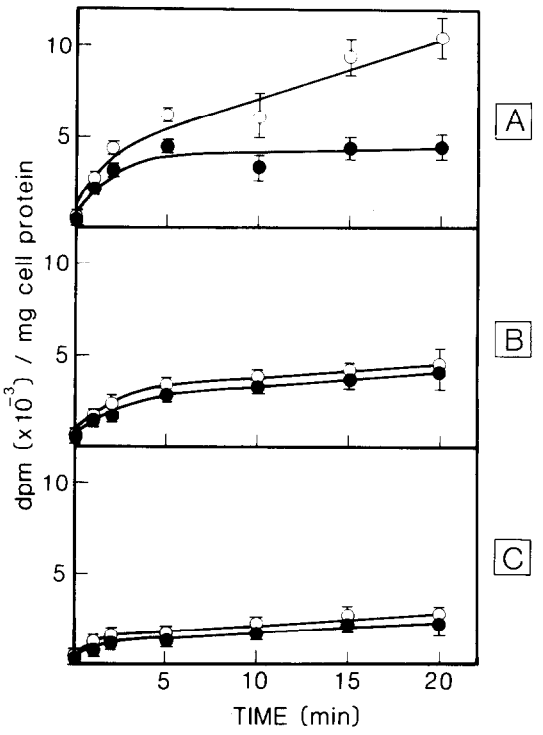

Fig.4. Erythroblasts (~300 μg protein) were incubated from 0–20 min at 37°C in 1 ml culture medium containing 10 μg/ml of ^{59}Fe-loaded, ^{3}H-labelled transferrin (spec. act. as in fig.1) in the absence (A) or in the presence of 30 mM methylamine (B) or 1 mM chloroquine (C). Mean results ± SD of 3 independent experiments are given: (o) ^{59}Fe uptake by the cells; (•) [^{3}H]transferrin uptake by the cells.

than ^{3}H. We have also investigated the effect of chloroquine and methylamine on the uptake of ^{59}Fe and ^{3}H during short term experiments (fig.4B,4C). In the presence of 10 mM methylamine, ^{59}Fe and ^{3}H are equally accumulated by the erythroblasts and in amounts comparable to the level reached by the ^{3}H-label in the absence of the drug; in the presence of 100 μM chloroquine, the accumulation of both isotopes is still comparable but further decreased by ~50%. On the other hand at 0°C, it should be noted that neither of these 2 drugs significantly affects the low temperature binding of transferrin to the cells (not shown).

4. Discussion

These data suggest that rat erythroblasts take up iron from transferrin through the presence of receptors specific for the protein and located at the plasma membrane. The number of receptors (~500 000/cell) is high but can be compared to data for reticulocytes [17] which indicate the presence of 300 000 receptors/cell; the K_a of the high affinity binding sites for transferrin is very close to published values (review [18]). At 37°C, these cells take up iron from transferrin in a continuous process saturable with the transferrin concentration. However, considering the amount of ^{3}H-label accumulated by the cells, and the absence of degradation of the protein, one has to conclude that, as for cultured fibroblasts [4], there is a mechanism whereby after transferrin binding and iron release, the iron-depleted transferrin is returned in an intact form to the extra-cellular medium. From the short-term kinetics, one can estimate that the interaction of transferrin with the erythroblasts takes place within ~5 min. At the saturating concentration of 100 μg/ml and during 6 h incubation, erythroblasts corresponding to 1 mg cell protein have taken up 7.4 ng ^{59}Fe which corresponds to the amount of iron bound to 5 μg transferrin . h^{-1} . mg cell protein^{-1}. Assuming the presence of 500 000 receptors/cell and 6×10^{6} erythroblasts/mg cell protein, each receptor must handle 12.5 transferrin molecules/h, i.e., 1 molecule every 5 min. This fits very well with the estimation made from the short-term kinetics.

The exact mechanism whereby transferrin iron is taken up by the erythroblasts is not known. As a first hypothesis, one can envisage that after binding of transferrin to its plasma membrane receptor, iron could be released, cross the membrane, and be incorporated into the cytosol. A second mechanism is similar to what we have proposed to explain the iron uptake in fibroblasts, and furthermore, it takes into account the morphological experiments [2,3], which indicated that in erythroid cells, transferrin is endocytosed within intracellular vesicles. It consists in receptor-mediated endocytosis of transferrin, release of iron after fusion of the endocytic vesicle with a lysosome, and recycling of the iron-depleted transferrin to the extracellular medium. The kinetic parameters are compatible with endocytosis and the effect of lysosomotropic agents provides strong arguments in favour of this mechanism.

Considering that receptor-mediated endocytosis of transferrin, release of iron within an intracellular acidic granule and recycling of the iron-depleted transferrin are probably involved in iron uptake by different cell types, this mechanism could be general. However, the

precise intracellular site where iron could be released from transferrin is not unequivocally established. There is considerable evidence in favour of lysosomes. The short term kinetics and the effect of lysosomotropic agents are compatible with the intralysosomal release of iron, and experimental evidence has been provided [19,20], showing that ligands tightly bound to the plasma membrane gain access to the lysosomes before being recycled back to the cell surface. However, at this stage one cannot rule out that most of the iron could be released from transferrin in another intracellular acidic granule and therefore bypass the lysosomes and their hydrolytic enzymes.

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