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TRANSFERRIN RECEPTORS AND IRON UPTAKE DURING ERYTHROID CELL DEVELOPMENT

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Experiments were performed to determine the level of transferrin receptors and rate of transferrin-bound iron uptake by various immature erythroid cell populations. Developing erythroid cells from the rat and mouse foetal liver at various stages of gestation were studied. In addition Friend leukaemic cells grown in culture were examined. The transferrin receptor level of Friend cells was similar to that of erythroid cells from the mouse foetal liver. During erythroid cell development the transferrin receptor level increased from about 300000 per cell at the early normoblast stage to reach a maximum of about 800000 per cell on intermediate normoblasts. Further maturation of intermediate normoblasts was accompanied by a decline in the number of transferrin receptors, reaching a level of 105000 in the circulating reticulocyte. The rate of iron uptake from transferrin during erythroid cell development was found to correlate closely with the number of transferrin receptors. In each of the immature erythroid cell populations studied the rate of iron uptake was about 36 iron atoms per receptor per hour. These results indicate that the level of transferrin receptors may be the major factor which determines the rate of iron uptake during erythroid cell development.

Introduction

Developing erythroid cells require large amounts of iron for the synthesis of haem. This is mediated by the presence of surface receptors which allow immature erythroid cells to obtain iron from transferrin, the plasma iron binding protein [1–3]. Current evidence suggests that the rate of iron uptake is maximal at the pronormoblast stage and that it decreases progressively during subsequent development [4–6]. The mechanism responsible for this change in rate of iron uptake during development is not known. It could be due to changes in the number of or binding properties of the transferrin receptors or to other regulatory factors such as the free intracellular haem concentration which has been proposed as the major regulator of iron uptake in reticulocytes [7]. In only one investigation has an attempt been made to correlate the changes in rate of iron uptake with the transferrin receptor levels at different stages of development [6]. However, in this work rabbit transferrin was used with erythroid cells derived from the spleens of mice treated with phenylhydrazine, and transferrin and iron uptake were measured separately. Hence it is difficult to interpret the quantitative significance of the results.

The aim of the present experiments was to investigate whether the changes in rate of iron uptake during erythroid cell development were accompanied by changes in the number and/or binding affinity of receptors for transferrin. From the results it should be possible to assess the significance of receptor function as a regulator of iron uptake by erythroid cells. The experiments

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were performed using erythroid cells from rat and mouse foetal liver and the homologous transferrin. Foetal livers from animals at various gestational ages were used to obtain populations of erythroid cells at different stages of development. Additional studies were carried out on foetal rat liver cells which had been allowed to undergo maturation in vitro and on Friend erythroleukaemia cells. The measurements were made using transferrins labelled with ^{59}Fe and ^{125}I so that iron uptake and receptor numbers and affinity could be determined simultaneously on each population of cells. The methods were based on those developed for reticulocytes [8] with modification to ensure measurement of specific transferrin receptors only [9].

Materials and Methods

Cell preparation. Rat and mouse foetal liver erythroid cells were obtained by dissection of foetal livers from 13 to 15 day gestational animals which had been killed by cervical dislocation. The livers were disaggregated in Hank's balanced salts solution [10] by repeated aspiration using a Pasteur pipette. A single cell suspension was obtained by passing the cell mixture through a fine mesh filter. The final cell preparation contained only 2% hepatocytes. Cells were washed twice in Hanks' solution and kept at 4°C for no longer than 30 min before use. Sterile precautions were taken when the cells were to be cultured. Rat reticulocytes were obtained by performing heart puncture on animals which had been bled three times in the preceding week to induce reticulocytosis. Reticulocyte-rich blood was collected into heparinized tubes and washed three times in 0.15 M NaCl. The reticulocyte count was about 40% in all experiments.

Cell culture. The Friend cell clone 707T used in this study was obtained from Dr. David Conkie, Beatson Institute for Cancer Research, Glasgow, Scotland. The cells were maintained in Hams F12 medium (Flow Laboratories, McLean, VA, U.S.A.) supplemented with 10% foetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia). Friend cells were induced to differentiate by the addition of dimethyl sulphoxide (DMSO) at a final concentration of 2% to cultures at a density of about 100000 cells per ml. The cells were grown in the presence of DMSO for five days by which time about 80% of the cells stained for haemoglobin. Before use the Friend cells were washed twice in Hanks' solution and then resuspended in Hanks' solution containing 1% bovine albumin.

Erythroid cells from the 15-day gestation rat foetal liver were cultured in Hams F12 medium supplemented with 10% foetal calf serum for two days. Cells were cultured at an initial density of 3–6 million cells per ml.

Transferrin purification and labelling. Rat and mouse transferrins were purified from plasma by gel-filtration and ion-exchange chromatography using methods described previously [11]. The proteins migrated as single bands on cellulose acetate electrophoresis. The purified transferrins were labelled with ^{125}I and ^{59}Fe as described earlier [12]. The ^{59}Fe, together with carrier iron, was added to apotransferrin as the complex with nitrilotriacetate in an amount just sufficient to saturate the specific iron-binding sites of the protein. The protein was then labelled with ^{125}I and was dialysed against three changes of 0.15 M NaCl.

Cell incubation procedures. Samples of foetal liver erythroid cells, Friend cells and reticulocytes containing between 3 and 10 million cells in 100 μl of Hanks' solution were incubated with 0.3 ml of the double labelled transferrin in Hanks' solution. The concentration of transferrin ranged from 0.11 μM to 4.2 μM. Incubation was performed at 37°C for 30 min in a shaking water bath. By this time transferrin uptake had reached a plateau level or steady state but iron uptake was continuing in a linear fashion. At the end of the incubation period the cells were washed three times with 2 ml ice cold phosphate-buffered saline. After the second of these washes the cells were transferred to new tubes, washed a third time and finally lysed by the addition of distilled water. Radioactivity was then measured using a 3-channel gamma radiation counter. Cellular uptake of transferrin and iron were calculated from these results and from the specific activity of the ^{125}I-^{59}Fe-transferrin. Transferrin uptake was expressed as molecules of transferrin bound per cell when a steady state had been reached and iron uptake as the rate of uptake in atoms of iron per cell per 30 min.

Transferrin uptake by immature erythroid cells involves reversible binding to cell membrane receptors. Hence, the transferrin data were analysed by the method of Scatchard [13] to estimate the number of specific receptors on the cells (B_{max}), assuming a unimolecular interaction between the transferrin and receptor molecules, and the apparent association constant for the interaction ('K_a') as described earlier [8]. Also, the rate of iron uptake shows Michaelis-Menten type kinetics [14] allowing the data to be analysed by the Eadie-Hofstee method [15] to determine the maximum rate of iron uptake (V_{max}) and the K_m of the uptake process [16]. The interaction of iron-transferrin with receptors on intact cells is not a true equilibrium process since the transferrin released from the cells is in an iron-depleted form. Hence, the K_a values are not true association constants. They will be referred to as the 'apparent association constant' or 'K_a'.

Analytical methods. Cell counts were made using a haemocytometer. The percentage of haemoglobinized cells in induced Friend cell cultures was estimated after staining with benzidine/HCl [17]. Reticulocyte counts were made after staining blood smears with new methylene blue. Differential cell counts of foetal liver erythroid cells were made after staining fixed cytocentrifuge smears with Giemsa. The criteria used for classification were the same as those used previously [18]. At least 500 cells were counted in all cases.

Statistical analysis of data. Linear regression using the method of least squares was used to determine the maximal number of specific transferrin binding sites and their association constant. The Student's *t*-test was used to compare the mean from different groups of experimental data. Differences were considered statistically significant when the probability, P, was less than 0.05.

Results

Rat foetal liver erythroid cells

The changes in cellular composition of the erythroid cell population between days 13 and 15 of gestation are shown in Fig. 1. The composition of the 15 day foetal erythroid cell population cultured for 1 and 2 days is also shown in Fig. 1. Pronormoblasts and basophilic normoblasts comprise about 55% of the erythroid cell population in the 13-day foetal liver. The 13-day erythroid cell population proliferates and differentiates until by day 15 of gestation about 80% of the cells synthesize haemoglobin. Further in vitro maturation of the 15-day erythroid cell population resulted in 65% of the cells being enucleated after two days of culture. Thus, there was a general maturation of the erythroid cell population from one of early normoblasts to early reticulocytes during the period of in vivo and in vitro foetal liver development described above.

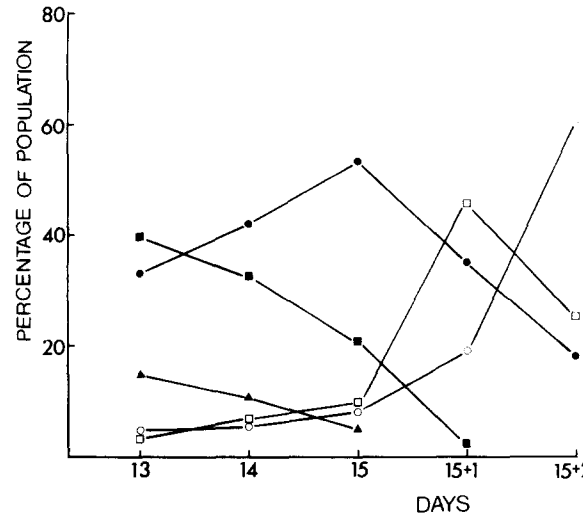

Fig. 1. Composition of the rat foetal liver erythroid cell population between days 13 and 15 of gestation and of cultured 15-day gestation foetal liver erythroid cells. ▲——▲, pronormoblasts; ■——■, basophilic normoblasts; ●——●, polychromatic normoblasts; □——□, orthochromatic normoblasts; ○——○, reticulocytes and red blood cells.

The foetal liver erythroid cell populations were incubated with varying concentrations of transferrin to determine the average cellular transferrin receptor level. Uptake of transferrin by the 13, 15 and 15 + 2 day foetal liver erythroid cells is shown in Fig. 2a, together with the corresponding Scatchard plots for this data (Fig. 2b). Saturation of the high affinity transferrin binding sites was found to occur at a transferrin concentration of about 2 μM in all the erythroid cell populations studied. Scatchard analysis of the transferrin uptake data for each of the foetal liver erythroid cell populations was performed to determine the num-

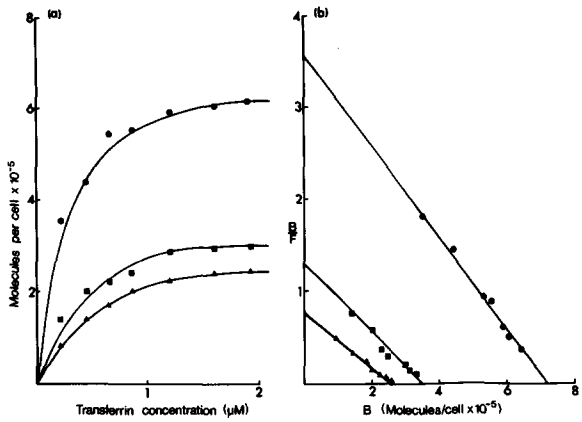

Fig. 2. Uptake of transferrin (a) and the corresponding Scatchard plots (b) by rat foetal liver erythroid cells. ■——■, 13-day gestation; ●——●, 15-day gestation; ▲——▲, 15-day gestation cells cultured for 2 days. The cells were incubated with transferrin for 30 min at 37°C.

ber of specific transferrin receptors (Fig. 3). Because the erythroid cell populations are heterogeneous with regard to the stage of development, the receptor level obtained is an average value for all the cell types present. Some cells in the population

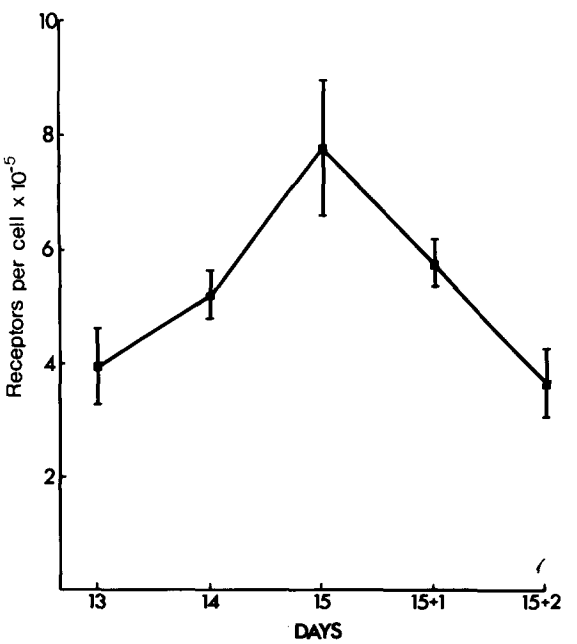

Fig. 3. Transferrin receptor levels of rat foetal liver erythroid cells from various stages of foetal liver development. Results shown are the mean and standard deviation from at least three experiments per cell population.

have more transferrin receptors than the average while others have less. Analysis of the data by the Student's *t*-test showed that each of the changes in transferrin receptor level of the erythroid cell populations during in vivo and in vitro foetal liver development was statistically significant ($P < 0.05$).

A 2-fold increase in the average cellular transferrin receptor level was observed between days 13 and 15 of gestation. During this period the erythroid cell population developed from one comprising of mostly early normoblasts to one consisting mainly of intermediate normoblasts in the 15-day gestational foetal liver. Further in vitro maturation of the 15-day foetal liver erythroid cells was found to result in a rapid decline in the level of transferrin receptors. The average cellular transferrin receptor level decreased from almost 800000 receptors per cell in the 15-day foetal liver to about 350000 receptors per cell after two days of culture. There were no significant differences in the values for the apparent association constant of the receptor for transferrin ('K_a'). The 'K_a' value ranged from $0.25 \cdot 10^7$ to $0.45 \cdot 10^7$ 1/mol. Circulating rat reticulocytes were found to have 105 000 transferrin receptors per cell (Table I).

Mouse foetal liver erythroid cells and Friend cells

The transferrin receptor level of erythroid cells from the mouse foetal liver and Friend cells were estimated as described for the rat foetal liver erythroid cells. Uninduced Friend cells were studied both in the stationary and logarithmic growth phases. Induced Friend cells were obtained by growth in the presence of 2% DMSO for 5 days. The 13-day mouse foetal liver erythroid cell population consisted of 49% early normoblasts which by day 15 of gestation had decreased to 20% of the population. Induced Friend cells were similar to the 15-day foetal liver erythroid cell populations with regard to the stage of development. About 80% of the cells in both populations were haemoglobin-synthesizing, polychromatic normoblasts.

The transferrin receptor level increased from about 300000 per cell to 500000 per cell during development of foetal mouse liver erythroid cells between days 13 and 15 of gestation and during DMSO-induced differentiation of Friend cells (Ta-

TABLE I

TRANSFERRIN RECEPTORS AND IRON UPTAKE

The results were calculated as described in the text from the transferrin and iron uptake data. Also given is the ratio of the maximum rate of iron uptake to the maximum transferrin uptake by the cells, $V_{max}(Fe)/B_{max}(TN)$.

Immature erythroid cell population	Transferrin uptake		Iron uptake		$V_{max}(Fe)/B_{max}(TN)$
	B_{max} (molecules/cell) ($\times 10^{-5}$)	'K_a' (M^{-1})($\times 10^{-7}$)	V_{max} (atoms/cell per 30 min) ($\times 10^{-5}$)	K_m (M)($\times 10^{6}$)	
14-day rat F.L.	5.28±0.40	0.36±0.05	118±16	0.49±0.11	22.3
15-day rat F.L.	7.75±1.14	0.42±0.22	170±16	0.71±0.20	21.8
15+1 day rat F.L.	5.89±0.20	0.45±0.04	99±21	0.61±0.26	16.8
15+2 day rat F.L.	3.59±0.46	0.33±0.15	55± 8	0.36±0.10	15.3
Rat reticulocytes	1.05±0.18	0.42±0.06	20± 4	0.80±0.24	19.0
13-day mouse F.L.	3.35±0.90	0.30±0.11	55±10	0.71±0.28	16.3
15-day mouse F.L.	5.23±0.47	0.44±0.13	72±19	0.76±0.15	14.0
Uninduced F.L.C.	2.62±0.56	0.25±0.09	51± 8	0.67±0.21	19.2
Induced F.L.C.	4.85±0.54	0.31±0.20	75±12	1.02±0.41	15.6
Mouse reticulocytes [a]	0.86±0.20	0.42±0.11	17± 3	0.38±0.10	19.8

[a] From Yeoh and Morgan [9].

ble I). The increases in transferrin receptor level in both cases were significant to a P value of less than 0.005. 'K_a' values of the transferrin receptors in all four cell populations were similar to those of foetal rat liver erythroid cells. Uninduced Friend cells from cultures which had just reached the stationary growth phase were found to have about half the level of transferrin receptors as cells which were from cultures in the logarithmic growth phase. Stationary phase cells had an average of 123000 transferrin receptors per cell while cells from the logarithmic growth phase had 262000 receptors per cell.

Iron uptake by immature erythroid cell populations

The uptake of iron from transferrin by immature erythroid cells is a saturable process and shows Michelis-Menten type kinetics (Fig. 4a). The uptake data was analyzed by the Eadie-Hofstee method to determine the maximal rate of iron uptake (V_{max}) and the K_m value of the uptake process (Fig. 4). Results for rat and mouse foetal liver erythroid cells, Friend cells and rat reticulocytes are shown in Table I. Results obtained from a previous study on mouse reticulocytes [9] are also given. As with the transferrin receptor level, estimations of the iron uptake rates are an average for all the cell types present. The increase in iron uptake during development of the mouse foetal liver erythroid cells from day 13 to 15 of gestation was not significant ($P < 0.05$). However the changes in iron uptake during DMSO induced differentiation of Friend cells and during in vivo and in vitro development of foetal rat liver

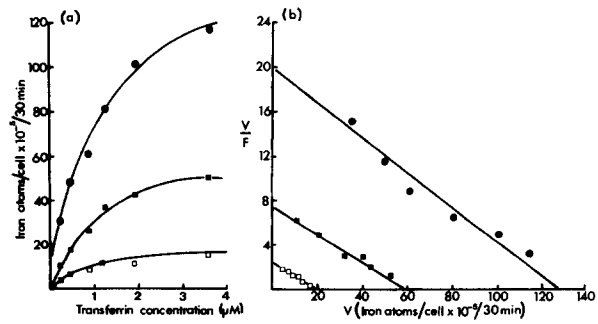

Fig. 4. Iron uptake from transferrin (a) and the corresponding Eadie-Hofstee plots (b), V represents the rate of iron uptake and F the concentration of free transferrin in the incubation solution. □——□, rat reticulocytes; ■——■, 13-day mouse foetal liver erythroid cells; ●——●, 15-day rat foetal liver erythroid cells. The cells were incubated with transferrin for 30 min at 37°C.

erythroid cells were significant to a P value of less than 0.005.

Table I also shows the ratio of iron uptake per transferrin receptor for each of the erythroid cell populations. This value is a measure of the efficiency of the transferrin receptors in allowing the immature erythroid cells to accumulate iron from transferrin. If it is assumed that both iron atoms are removed from the transferrin molecule then the iron uptake per transferrin receptor per 30 min is equivalent to the turnover of transferrin molecules per receptor per hour. There was relatively little difference in this value for all of the cell populations studied. The mean value and standard deviation for the rate of iron uptake per transferrin receptor by the ten different immature erythroid cell populations was 18.0 ± 2.8 iron atoms per receptor per 30 min.

Discussion

The results of the present experiments using rat and mouse foetal liver erythroid cells and Friend cells show that the transferrin receptor level increases during erythroid cell development from the early normoblast stage to the intermediate normoblast stage. The results also indicate that immature rat erythroid cells have more transferrin receptors than immature mouse erythroid cells at the same stage of development. Friend cells were found to have similar numbers of transferrin receptors to normal mouse erythroid cells from the foetal liver. In the rat, further maturation of the intermediate normoblasts was accompanied by a rapid decline in the level of transferrin receptors, finally reaching a value of about 105000 per cell in reticulocytes. Because the erythroid cell populations used were heterogeneous with regard to the stage of development, definitive values for transferrin receptor levels at each stage of development could not be obtained. However, it can be concluded that there was some increase in transferrin receptor levels during development from the early to intermediate normoblast stages of development. A technique which allows observation and measurement of transferrin binding at the single cell level is required to give quantitative values for each stage of development. Quantitative fluorescence microscopy is currently being used to achieve this.

Friend cells were shown in this study to have similar transferrin receptor levels to normal immature erythroid cells from the mouse foetal liver. Although the transferrin receptor level of induced Friend cells observed in this study was the same as that found by Yeoh and Morgan [9], the uninduced Friend cell receptor level was much higher. This difference is probably due to the use of cells which were in the stationary growth phase in the studies by Yeoh and Morgan. It was found in the present investigation that uninduced cells from the logarithmic growth phase possess about twice the level of transferrin receptors as uninduced Friend cells from the stationary growth phase. A decrease in transferrin receptor level following the transition of uninduced Friend cells from the logarithmic to the stationary growth phases has been observed previously [19]. Larrick and Cresswell [20] have observed a similar effect of cell density on transferrin binding by cultured lymphocytes.

This study using nine different immature erythroid cell populations demonstrates a close correlation between the rate of iron uptake and the number of transferrin receptors. The stages of erythroid development examined included early normoblasts (13-day rat and mouse foetal liver and uninduced Friend cells), intermediate normoblasts (15-day rat and mouse foetal liver and induced Friend cells) and reticulocytes. Iron uptake increased between the early and intermediate normoblast stages of development and then decreased during maturation to the reticulocyte. These changes are similar to the changes in transferrin receptor levels during development. About 36 iron atoms per hour per transferrin receptor were taken up by each of the erythroid cell populations. It can be concluded from these results that the number of transferrin receptors is a major determinant of the rate of iron uptake during erythroid cell development.

The values for the apparent association constant of the transferrin receptor of the immature erythroid cell populations examined were in the range $0.25 \cdot 10^7$ to $0.45 \cdot 10^7$ l/ml. No significant differences were found between the populations. Although these values do not represent the true equilibrium constant of association of the receptors and transferrin they are probably some function of this constant. If this is so it indicates that

little or no change in the binding affinity of the receptors occurs during erythroid cell development. Similarly, the K_m values for iron uptake were relatively constant. Hence, it is unlikely that the changes in rate of iron uptake during development are a result of altered affinity of the receptors for transferrin or of the K_m values of the transport process. Instead, they appear to be largely determined by the number of receptors per cell, as concluded above.

The rate of iron uptake by human bone marrow erythroid cells has been estimated previously by autoradiography [4,5]. In these studies it was found that the highest uptake was in the proerythroblasts and that a progressive decrease in uptake accompanied maturation to the reticulocyte. In the present study maximal iron uptake was found in the intermediate normoblasts. Species difference and the different method used in the earlier studies may account for maximal iron uptake being observed in proerythroblasts.

Nunez et al. [6] measured transferrin binding and ^{59}Fe uptake from transferrin by mouse spleen erythroid cells and also concluded that a progressive decrease in iron uptake occurs during development. However, in these studies most of the measurements were made with a non-homologous system, rabbit transferrin with mouse spleen erythroid cells, using concentrations which are well above the saturation concentration of transferrin receptors observed in the present study and in other investigations [21,9]. It is likely that the high transferrin receptor levels observed by Nunez et al. were due to the inclusion of non-specific binding sites in their measurements. The transferrin receptor levels determined by these workers were about 10-fold greater than those observed in this study. Non-specific transferrin binding sites are probably non-functional and this may account for the very low iron uptake/transferrin receptor ratios determined in the study by Nunez et al.

The present study shows that the rate of iron uptake by erythroid cells at various stages of development is closely correlated to the level of specific transferrin receptors. Maximal iron uptake and level of transferrin receptor occur at the intermediate normoblast stage of development. The results from this study suggest that in developing erythroid cells the level of transferrin receptor is a major regulatory factor in the control of iron uptake. Further investigation is required to determine whether other regulatory factors such as intracellular free haem levels are also operative during earlier stages of development than the reticulocyte.

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