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The effect of desferrioxamine and ferric ammonium citrate on the uptake of iron by the membrane iron-binding component of human melanoma cells

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The effect of ferric ammonium citrate (FAC) and desferrioxamine (DFO) on membrane iron and transferrin uptake have been investigated using SK-MEL-28 human melanoma cells which express the membrane-bound transferrin homologue, melanotransferrin, at high concentrations. Exposure of melanoma cells to DFO increased membrane non-Tf-bound Fe uptake (putative melanotransferrin Fe-binding sites), suggesting upregulation of the membrane Fe-binding component. However, exposure to FAC did not result in down-regulation. Indeed, an increase in non-Tf-bound membrane Fe was apparent. Results suggested that non-Tf-bound membrane Fe uptake occurred by two processes corresponding to the specific and non-specific mechanisms of Fe uptake from Tf described previously (Richardson, D.R. and Baker, E. (1990) Biochim. Biophys. Acta 1053, 1–12).

Introduction

We are investigating the processes of Fe uptake by the melanoma cell and particularly the role of the membrane-bound, transferrin (Tf) homologue p97, or melanotransferrin (MTf), in these processes. Melanotransferrin has many properties in common with serum transferrin (Tf), including a 37–39% sequence homology and the ability to bind Fe(III) from citrate Fe-59 complexes [1–3]. Previous studies by the authors have identified a membrane Fe-binding molecule in human SK-MEL-28 melanoma cells which bound Fe from diferric Tf and had characteristics consistent with MTf [4]. However, chase experiments demonstrated that the membrane-bound Fe was not metabolised by the cell, suggesting that it may have no role in Fe uptake [5]. Further work demonstrated that MTf had no role in uptake of Fe from small molecular weight chelates [6], as had been previously suggested [7].

Apart from the role of MTf in Fe uptake, the mechanisms of Fe uptake from Tf in melanoma cells were also investigated. Two main processes of Fe uptake were found: (A) a specific process consistent with receptor-mediated endocytosis which saturated at a Tf concentration of 0.01 mg/ml, and (B) a non-specific process which increased in importance after saturation of the TfR and was consistent with the release of Fe from Tf at the cell surface by an oxidoreductase [4,8,9].

Since TfR expression appears to be controlled by intracellular Fe concentration [10–13] it was deemed worthwhile to investigate whether intracellular Fe concentration could also regulate the expression of the membrane Fe-binding molecule (putative MTf sites) found in melanoma cells.

Ferric ammonium citrate (FAC) when incubated with cells results in an accumulation of cell-associated Fe and ferritin [14,15] resulting in down regulation of the TfR [10–12,16,17]. Desferrioxamine has a high specificity for Fe(III) ion [18] and can chelate intracellular Fe pools [19,20] which may regulate TfR biosynthesis [21]. Hence, FAC and DFO were well suited to modulate cellular Fe levels.

Abbreviations: BSA, bovine serum albumin; BSS, balanced salt solution; DFO, desferrioxamine; FAC, ferric ammonium citrate; Fe, iron; gPR, gram of protein; MEM, minimum essential medium; MTf, melanotransferrin; Tf, transferrin.

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Exposure of melanoma cells to DFO increased membrane non-Tf-bound Fe uptake, suggesting up-regulation of the membrane Fe-binding component. However, exposure to FAC did not result in down-regulation. Indeed, an increase in non-Tf-bound membrane Fe was apparent. Results suggested that membrane Fe uptake occurred by two processes corresponding to the specific and non-specific mechanisms of Fe uptake described previously [4,8,9].

Materials and Methods

Chemicals

Iron-59 (as ferric chloride in 0.1 M HCl) and iodine-125 (as sodium iodide) were purchased from Amersham International, Amersham, Bucks, UK. Pronase was purchased from Boehringer Mannheim, Mt. Waverley, Vic., Australia. Eagle's modified minimum essential medium (MEM) as Autopow and fetal calf serum (FCS) were supplied by Flow Laboratories, Annandale, N.S.W., Australia. Penicillin (Crystapen-Benzylpenicillin sodium B.P.) was obtained from Glaxo Australian Pty. Ltd., Boronia, Vic., Australia. Bovine serum albumin (BSA; 98% pure, fatty acid free), human apoTf, L-glutamine and *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (Hepes) was obtained from Sigma Chemical Co., St. Louis, MO, USA. Non-essential amino acids (100 × concentrate) and trypsin-versene solution (1 ×) were obtained from Commonwealth Serum Laboratories, Melbourne, Vic., Australia. Balanced salt solution (BSS) was prepared by the method of Hanks and Wallace [22]. Ferric ammonium citrate (17.5% Fe) was from Aldrich Ltd. Desferrioxamine was from Ciba-Geigy Pharmaceutical Co. All other chemicals were of analytical reagent quality.

Protein purification and labelling

Human apoTf was prepared and labelled with Fe-59 and iodine-125 using the methods of Hemmaplardh and Morgan [23] and McFarlane [24] as described previously [4].

Cell culture

The human melanoma cell line, SK-MEL-28 (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA) was used as these cells have the highest concentration of MTf of all cell types studied [25]. Cells were grown and subcultured as described previously [4]. Procedures used to check cell viability and differentiation of the cell line were also the same as described previously [4].

Experimental procedure-uptake of iron and transferrin after exposure to ferric ammonium citrate and desferrioxamine

Ferric ammonium citrate was used at two concentrations, 5 μg/ml and 150 μg/ml, corresponding to Fe concentrations of approximately 0.9 μg/ml and 26 μg/ml, respectively. The higher Fe concentration of 26 μg/ml was chosen as concentrations in this range increase intracellular Fe and decrease TfR expression without adverse affect [10,12,15,17].

Desferrioxamine was used at a concentration of 0.5 mM, at which concentration there was no adverse effect on cells as judged by morphological criteria, the release of LDH from the cells and the uptake of [^{3}H]leucine. Desferrioxamine enters the cell rapidly but leaves slowly, resulting in cellular accumulation [20]. Initial experiments demonstrated the accumulation of DFO within the cell and a washing procedure was used to deplete the cells of DFO before measuring Tf and Fe uptake (see below).

The DFO (0.5 mM) or FAC (5 or 150 μg/ml) was added to MEM containing non-essential amino acids (1%), BSA (5 mg/ml) and Hepes (20 mM; pH 7.4). This medium was prewarmed and gassed with 5% CO_2 before incubation with the cells for 20 h at 37 °C. The medium was then removed and the cell monolayer depleted of DFO or FAC by three separate 45 min incubations in MEM at 37 °C before adding prewarmed and gassed MEM containing doubly labelled Tf (0.001 mg/ml to 0.06 mg/ml) which was incubated with the cells for 2 h. In no instance was FCS added to the medium containing radiolabelled Tf. The amount of Fe or Tf internalised by the cells was measured by incubation with pronase (1 mg/ml) for 30 min at 4 °C, as described previously [4].

Results

The effect on the uptake of iron and transferrin of prewashing the cell monolayer with minimum essential medium, 30% fetal calf serum or ferric ammonium citrate prior to the addition of labelled transferrin

Initial experiments were designed to investigate whether the membrane Fe-binding molecule (putative MTf sites) described previously [4] could bind Fe-56 added to the medium as FAC and prevent membrane Fe-59 uptake. In these experiments cells were washed with MEM, 30% FCS in MEM or MEM containing FAC (0.5–25 μg/ml) using three separate 45 min incubations at 37 °C before adding MEM containing doubly labelled Tf (0.1 mg/ml), 1% non-essential amino acids, BSA (5 mg/ml) and Hepes (20 mM; pH 7.4) for 2 h. Subsequent procedures were performed as described previously [4].

Compared to when the cells were not washed (NW), membrane non-Tf-bound Fe uptake was significantly ($P < 0.0005$) enhanced by washing the cells with MEM prior to the addition of labelled Tf (Fig. 1). In contrast, washing cells with 30% FCS in MEM compared to not washing (NW) at all, significantly ($P < 0.025$) reduced non-Tf-bound Fe uptake (Fig. 1). However, when the

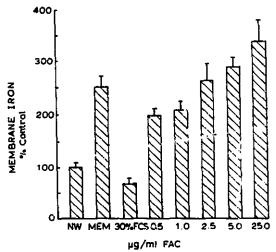

Fig. 1. The effect on non-transferrin-bound membrane iron of washing or not washing (NW) the melanoma cell monolayer with minimum essential medium (MEM), 30% fetal calf serum (30% FCS) or ferric ammonium citrate (0.5–25 μg/ml) using three 45-min incubations. After this procedure the medium was removed and the cells reincubated with ^{59}Fe-^{125}I-transferrin (0.1 mg/ml) for 2 h at 37°C and then treated with pronase (1 mg/ml) for 30 min at 4°C. Non-transferrin-bound membrane iron uptake was calculated by assuming that each transferrin molecule on the membrane bound two iron atoms. This was then subtracted from the total membrane iron uptake. Results are expressed as a percentage of that obtained when the cell monolayer was not washed (NW). Results are means ± S.E. (two experiments; nine or ten determinations).

cell monolayers were washed with MEM containing FAC (0.5–25 μg/ml), no decrease in non-Tf-bound Fe uptake occurred. In fact, at a FAC concentration of 25 μg/ml, there was a significant ($P < 0.025$) increase in non-Tf-bound Fe uptake (Fig. 1). These changes were studied further by examining the effect of incubation with MEM, 30% FCS, DFO (0.5 mM) and FAC (5 μg/ml and 150 μg/ml) for 20 h, before incubation for 2 h with ^{59}Fe-^{125}I-Tf at a number of Tf concentrations.

The effect of transferrin concentration on iron and transferrin uptake into the membrane after exposure to desferrioxamine, ferric ammonium citrate, 30% fetal calf serum and minimum essential medium (control)

(1) Membrane molar ratio of iron: transferrin

Previous studies [4,5] demonstrated that when melanoma cells were grown in MEM containing 30% FCS an increase in the membrane molar ratio of Fe to Tf from about 2.0 to 2.6 was detected after incubation with doubly labelled Tf, suggesting the presence of a membrane Fe-binding molecule. Hence, the effect on the membrane molar ratio of Fe to Tf of exposing cells to MEM, FAC and DFO for 20 h was of interest.

For all Tf concentrations studied, and for all conditions investigated (apart from cells treated with 30% FCS alone), the membrane molar ratio of Fe to Tf varied between 3 and 5.25 (Fig. 2). When cells were incubated with 30% FCS alone, the membrane molar ratio of Fe:Tf varied from 2.60 to 3.30 at Tf concentrations of 0.001–0.06 mg/ml (Richardson and Baker, 1990). This was generally less than that obtained when the cells were incubated for 20 h in MEM with or without DFO or FAC (Fig. 2).

The amount of membrane-bound Fe-59 in excess of that due to Tf-bound Fe was estimated by subtracting from the observed amount of Fe-59 at 37°C that calculated assuming two atoms of Fe per membrane-bound Tf, as described previously [4,5]. Fig. 3 illustrates the membrane Fe observed and calculated after an incubation period of 20 h with MEM. It should be noted for cells treated with 30% FCS, FAC, DFO or MEM, that in all cases the amount of membrane-bound Fe observed was significantly greater ($P < 0.05$–0.0005) than that calculated.

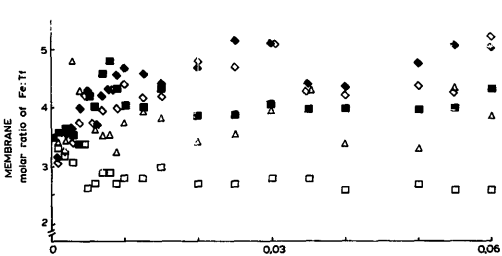

Fig. 2. The effect of transferrin concentration on the molar ratio of iron/transferrin on the cell membrane after exposure of melanoma cells to minimum essential medium (MEM; △), desferrioxamine (DFO; 0.5 mM; ■), ferric ammonium citrate (FAC) at a concentration of 5 μg/ml (◇) and 150 μg/ml (◆) and 30% fetal calf serum (30% FCS; □) for 20 h. The cells were then washed using three separate 45-min incubations in MEM and reincubated with ^{125}I-^{59}Fe-transferrin (0.001–0.06 mg/ml) for 2 h at 37°C and then treated with pronase (1 mg/ml) for 30 min at 4°C. Results are means of three experiments (DFO), four experiments (MEM), five experiments (FAC) and six experiments (30% FCS).

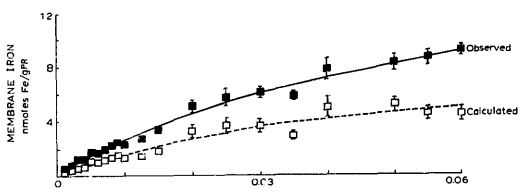

Fig. 3. The effect of transferrin concentration on iron uptake into the membrane compartment after exposure of melanoma cells to minimum essential medium (MEM) for 20 h. The cells were then washed using three separate 45-min incubations in MEM and reincubated with ^{125}I-^{59}Fe-transferrin (0.001–0.06 mg/ml) for 2 h at 37 °C and then treated with pronase (1 mg/ml) for 30 min at 4 °C. Both the observed (total) membrane Fe uptake (■) and calculated membrane iron uptake (□; estimated by assuming that each Tf molecule in the membrane compartment has two iron atoms bound) have been plotted for comparison. Results are means ± S.E. of four experiments.

(II) Non-transferrin-bound membrane iron

The difference between the observed and calculated membrane Fe (i.e., non-Tf-bound Fe) varied in a biphasic manner (Fig. 4) which had inflection points at Tf concentrations of approximately 0.015–0.02 mg/ml. The biphasic nature of the curves may be due to incorporation of Fe by the two separate processes of Fe uptake from Tf described previously [4]. The rates of non-Tf-bound membrane Fe uptake were calculated from the two linear components between Tf concentrations of 0.001 and 0.015 mg/ml and from 0.02 to 0.06 mg/ml (Table I). Since the slopes of the Fe uptake for all treatments were greater between Tf concentrations of 0.001 and 0.015 mg/ml than from 0.02 to 0.06 mg/ml (Fig. 4; Table I), it can be suggested that membrane non-Tf-bound Fe uptake appeared to occur more efficiently from the specific mechanism than the non-specific mechanism of Fe uptake.

At Tf concentrations between 0.001 and 0.015 mg/ml the rate of non-Tf-bound membrane Fe uptake was greatest after incubation of cells with DFO for 20 h and lowest for cells incubated with 30% FCS (Table I). Only for cells exposed to DFO was the rate of Fe uptake between Tf concentrations of 0.001 and 0.015 mg/ml significantly ($P < 0.005$) greater than the corresponding control (i.e., MEM). The rate of non-Tf-

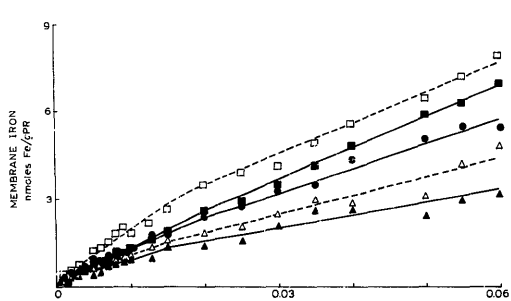

Fig. 4. The effect of transferrin concentration on the uptake of non-transferrin-bound iron by the cell membrane after exposure of melanoma cells to minimum essential medium (MEM; Δ), desferrioxamine (DFO; 0.5 mM; □), ferric ammonium citrate (FAC) at a concentration of 5 μg/ml (●) and 150 μg/ml (■) and 30% fetal calf serum (30% FCS; ▲) for 20 h. The cells were then washed using three separate incubations in MEM and reincubated with ^{125}I-^{59}Fe-transferrin (0.001–0.06 mg/ml) for 2 h at 37 °C and then treated with pronase (1 mg/ml) for 30 min at 4 °C. Results are means of four experiments (MEM), three experiments (DFO), 5 experiments (FAC) and six experiments (30% FCS).

TABLE I

The rate of non-transferrin-bound membrane iron uptake between transferrin concentrations of 0.001 and 0.015 mg/ml and 0.02 and 0.06 mg/ml.

Non-transferrin-bound membrane iron was calculated assuming that each transferrin molecule on the membrane had two iron atoms bound. The calculated membrane iron uptake was then subtracted from that observed. Results are means ± S.E. (number of experiments).

Treatment	Rate of Fe uptake (10^{-7} moles Fe/gPR per mg ml^{-1} Tf per 2 h)	
[Tf]:	0.001–0.015 mg/ml	0.02–0.06 mg/ml
MEM	0.93 ± 0.12 (4)	0.60 ± 0.10 (4)
DFO (0.5 mM)	1.64 ± 0.10 (3) [a]	1.02 ± 0.05 (3) [b]
FAC (5 μg/ml)	1.29 ± 0.17 (5)	0.85 ± 0.16 (5)
FAC (150 μg/ml)	1.26 ± 0.15 (5)	1.11 ± 0.10 (5) [c]
30% FCS	0.73 ± 0.08 (6)	0.39 ± 0.05 (6) [d]

[a] Significantly different ($P < 0.005$) from the MEM control.
[b] Significantly different ($P < 0.025$) from the MEM control.
[c] Significantly different ($P < 0.01$) from the MEM control.
[d] Significantly different ($P < 0.05$) from the MEM control.

bound membrane Fe uptake between Tf concentrations of 0.02 and 0.06 mg/ml was significantly ($P < 0.05$) less than the control when the cells were treated with 30% FCS, and significantly greater than the control when the cells were treated with DFO ($P < 0.025$) or 150 μg/ml FAC ($P < 0.01$; Table I).

Examining membrane Fe uptake quantitatively (Fig. 4), the amount of non-Tf-bound membrane Fe was significantly ($P < 0.0005$) greater in cells treated with DFO compared to MEM for all Tf concentrations, suggesting up-regulation of the Fe-binding component. However, in cells treated with FAC (5 or 150 μg/ml), down-regulation was not observed (Fig. 4). Indeed, at Tf concentrations between 0.001 and 0.01 mg/ml, there was no significant ($P > 0.05$) difference in the amount of non-Tf-bound Fe present when comparing cells exposed to MEM (control) or FAC (5 or 150 μg/ml; Fig. 4). This suggested no change in the expression of the membrane Fe-binding component. However, as the Tf concentration is increased past the saturation of the specific TfR at 0.01 mg/ml, the amount of non-Tf-bound Fe present in the membrane was significantly greater ($P < 0.05$–0.0005) in the presence of FAC at 5 or 150 μg/ml when compared to MEM alone. Above a Tf concentration of 0.0125 mg/ml, the amount of non-Tf-bound Fe becomes greater for cells treated with FAC at 150 μg/ml than at 5 μg/ml. This difference was significant ($P < 0.05$) as the Tf concentration is raised towards 0.06 mg/ml (Fig. 4). The amount of non-Tf-bound membrane Fe in cells exposed to 30% FCS alone was less than that found when cells were exposed to MEM with or without DFO or FAC for 20 h, especially at Tf concentrations above 0.015 mg/ml (Fig. 4). The effect of FCS of depressing membrane non-Tf-bound Fe uptake has been noted previously [4].

Discussion

If the membrane-bound, Fe-binding molecule of melanoma cells has a role in Fe metabolism, changing the intracellular Fe concentration may also change its expression.

(I) Washing experiments

Interestingly, non-Tf-bound membrane Fe uptake (putative MTf Fe uptake) occurred to a much greater extent if the cells were washed by preincubation with MEM prior to the addition of labelled Tf (Fig. 1). This suggested that while cells were growing in 30% FCS the membrane Fe-binding sites may become saturated with Fe-56, while incubation in essentially Fe-free MEM resulted in the metabolism of Fe-56-bound to the molecule, generating Fe-free sites capable of binding Fe-59 from Tf. To test this hypothesis the cells were preincubated with MEM containing FAC prior to the addition of ^{59}Fe-^{125}I-Tf in an attempt to saturate the Fe-binding sites and prevent or slow Fe-59 uptake from Tf. Indeed, MTf can chelate Fe from citrate-Fe(III) complexes [1]. However, exposure to MEM containing FAC did not decrease non-Tf-bound Fe uptake. Indeed, washing the cells with MEM containing FAC (25 μg/ml) resulted in a significant increase in membrane non-Tf-bound Fe uptake (Fig. 1). This suggested that the membrane Fe-binding molecule may only bind Fe released from Tf, as previously demonstrated [4].

Alternatively, washing the cells in MEM prior to adding labelled Tf may remove components in FCS which may induce redistribution of membrane Fe-binding sites. This is supported by previous work which demonstrated that FCS decreased membrane non-Tf-bound Fe-59 uptake [4]. It should be noted that FCS also has been shown to cause the redistribution of the TfR [26].

(II) Preincubation with desferrioxamine or ferric ammonium citrate

Exposure of melanoma cells to DFO for 20 h resulted in a significant increase in membrane non-Tf-bound Fe uptake at all Tf concentrations when compared to the MEM control, suggesting up-regulation of the membrane Fe-binding molecule (Fig. 4). Alternatively, it can be suggested that since DFO can stimulate Fe uptake by both the specific and non-specific mechanisms [8], Fe uptake by the membrane Fe-binding component may also be stimulated. This latter argument is supported by studies discussed below using FAC.

resulted in an increase in membrane Fe uptake when compared to the control, but only between Tf concentrations of 0.01 and 0.06 mg/ml. It should be noted that internalised Fe uptake is also only stimulated by FAC via the non-specific mechanism of Fe uptake between Tf concentrations of 0.01 and 0.06 mg/ml [8]. This observation suggested that Fe uptake by the membrane Fe-binding molecule may also be derived from the non-specific mechanism of Fe uptake. The biphasic nature of the non-Tf-bound Fe uptake curve (Fig 4) also suggested that Fe uptake by the membrane Fe-binding molecule occurred via two processes corresponding to the specific and non-specific processes of Fe uptake [4,8]. In addition, experiments with chelators [8] suggested that non-Tf-bound membrane Fe uptake also occurred via the two separate mechanisms of Fe uptake from Tf. In these experiments, hydrophilic, membrane-impermeable chelators were found to be effective at reducing membrane non-Tf-bound Fe uptake at high Tf concentrations where the non-specific mechanism of Fe uptake predominated. In contrast, at low Tf concentrations where Fe uptake via the specific mechanism predominated, membrane-impermeable chelators were found to be less effective at preventing membrane non-Tf-bound Fe uptake. Considering these observations cumulatively, stimulation of total cell Fe uptake from Tf by DFO and FAC [8] also resulted in enhanced uptake of membrane non-Tf-bound Fe-59.

Further studies examining the effects of FAC and DFO on Tf and Fe metabolism will be described in detail in subsequent publications (Richardson and Baker, unpublished data).

It was concluded that Fe uptake by the membrane Fe-binding molecule was derived from two processes corresponding to the specific and non-specific mechanisms of Fe uptake from Tf previously described in melanoma cells [4,8,9]. Further work is required to investigate how the membrane Fe-binding molecule acquires this Fe.

Acknowledgements

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