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Iron and gallium increase iron uptake from transferrin by human melanoma cells: further examination of the ferric ammonium citrate-activated iron uptake process

D.R. Richardson *

Heart Research Institute, Iron Metabolism and Chelation Group, 145 Missenden Rd, Camperdown, Sydney, NSW 2050, Australia

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Abstract

Previously we showed that preincubation of cells with ferric ammonium citrate (FAC) resulted in a marked increase in Fe uptake from both ^{59}Fe-transferrin (Tf) and ^{59}Fe-citrate (D.R. Richardson, E. Baker, J. Biol. Chem. 267 (1992) 13972–13979; D.R. Richardson, P. Ponka, Biochim. Biophys. Acta 1269 (1995) 105–114). This Fe uptake process was independent of the transferrin receptor and appeared to be activated by free radicals generated via the iron-catalysed Haber-Weiss reaction. To further understand this process, the present investigation was performed. In these experiments, cells were preincubated for 3 h at 37°C with FAC or metal ion solutions and then labelled for 3 h at 37°C with ^{59}Fe-Tf. Exposure of cells to FAC resulted in Fe uptake from ^{59}Fe-citrate that became saturated at an Fe concentration of 2.5 μM, while FAC-activated Fe uptake from Tf was not saturable up to 25 μM. In addition, the extent of FAC-activated Fe uptake from citrate was far greater than that from Tf. These results suggest a mechanism where FAC-activated Fe uptake from citrate may result from direct interaction with the transporter, while Fe uptake from Tf appears indirect and less efficient. Preincubation of cells with FAC at 4°C instead of 37°C prevented its effect at stimulating ^{59}Fe uptake from ^{59}Fe-Tf, suggesting that an active process was involved. Previous studies by others have shown that FAC can increase ferrireductase activity that may enhance ^{59}Fe uptake from ^{59}Fe-Tf. However, there was no difference in the ability of FAC-treated cells compared to controls to reduce ferricyanide to ferrocyanide, suggesting no change in oxidoreductase activity. To examine if activation of this Fe uptake mechanism could occur by incubation with a range of metal ions, cells were preincubated with either FAC, ferric chloride, ferrous sulphate, ferrous ammonium sulphate, gallium nitrate, copper chloride, zinc chloride, or cobalt chloride. Stimulation of ^{59}Fe uptake from Tf was shown (in order of potency) with ferric chloride, ferrous sulphate, ferrous ammonium sulphate, and gallium nitrate. The other metal ions examined decreased ^{59}Fe uptake from Tf. The fact that redox-active Cu(II) ion did not stimulate Fe uptake while redox-inactive Ga(III) did, suggests a mechanism of transporter activation not solely dependent on free radical generation. Indeed, the activation of Fe uptake appears dependent on the presence of the Fe atom itself or a metal ion with atomic similarities to Fe (e.g. Ga).

Keywords: Iron; Iron uptake mechanism; Melanoma cell; Transferrin

Abbreviations: FAC, ferric ammonium citrate; Fe, iron; MEM, Eagle's modified minimum essential medium; RME, receptor-mediated endocytosis; Tf, transferrin; TfR, transferrin receptor

* Fax: +61-2-9550-7502; E-mail: d.richardson@hri.org.au

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1. Introduction

Under physiological conditions virtually all iron (Fe) in the serum is bound to the Fe-binding protein, transferrin (Tf) (for reviews see [1,2]). In erythroid precursors such as reticulocytes, Tf donates its Fe to cells via its specific binding to the Tf receptor (TfR) on the cell membrane [2]. The Tf-TfR complex is then internalised by receptor-mediated endocytosis (RME) [1,2]. The Fe is released from Tf by a decrease in endosomal pH and is then transported through the membrane by Nramp2 (also known as the divalent metal ion transporter 1 (DMT1) or divalent cation transporter 1 (DCT1)) [3–5]. Apart from the well characterised RME process, a second mechanism of Fe uptake from Tf has been reported in rat hepatocytes [6–9], human melanoma cells [10,11], and human hepatoma cells [12]. In some of these studies an extracellular reductive process mediated by an oxidoreductase [13] was thought to be involved in the second mechanism [8,9]. In contrast, in other investigations an endocytotic or pinocytotic process was proposed [6,7,11]. Apart from these mechanisms, recent investigations have suggested that Fe uptake from Tf may also be mediated by the transferrin receptor 2 (TfR2), although the physiological significance of this molecule remains unclear [14,15].

Apart from Fe uptake from Tf, many cell types have also been shown to efficiently take up Fe from small molecular weight (M_r) complexes by a high capacity transport system ([16–19]; for review see [2]). Little is known concerning the molecular mechanisms involved in Fe uptake from low M_r complexes, most studies describing saturable Fe uptake with different but generally low K_m [17,18]. Preincubation of cells with the Fe complex ferric ammonium citrate (FAC) resulted in a marked increase in Fe uptake from Fe-nitrilotriacetic acid (Fe-NTA) complexes [19]. Results from this latter study suggested that this involved recruitment of transporters from an internal pool to the cell surface [19]. This observation was of importance, as ferric citrate is found in the serum of patients with Fe-overload disease [20], and may contribute to Fe accumulation within the liver and other organs [2].

Apart from the effect of FAC on stimulating Fe uptake from low M_r complexes, it was also of interest to find that incubation of melanoma cells with FAC had two effects on Fe uptake from Tf [21]. First, at Tf concentrations up to saturation of the TfR, Fe uptake from Tf was decreased due to the downregulation of the specific Tf-binding site. Second, at Tf concentrations higher than that required for saturation of the TfR, a marked increase in Fe uptake occurred [21]. A more recent study demonstrated that this latter process was not mediated by the TfR, and that ^{59}Fe uptake from ^{59}Fe-citrate could also be markedly enhanced after preincubation with FAC [22]. The addition of a range of free radical scavengers to FAC markedly depressed FAC-activated Fe uptake from Tf and Fe-citrate, whereas when these agents were added to control cells they had no effect [22]. The addition of hydrogen peroxide or superoxide-generating agents to FAC also stimulated Fe uptake, whereas little effect was found when these agents were added to medium alone [22]. These results suggested that activation of the FAC-stimulated Fe uptake mechanism was caused by the production of hydroxyl radicals via the Fe-catalysed Haber-Weiss reaction. It was hypothesised that this Fe uptake process may represent a defense mechanism against oxidant stress generated in the presence of low M_r complexes [22]. Additional studies using primary cultures of hepatocytes demonstrated that there was a marked increase in Fe uptake from both Tf and Fe-citrate after incubation with FAC [23]. These data may help explain the loading of hepatocytes with Fe that occurs in Fe-overload disease despite marked downregulation of the TfR [23].

In the present study we have further characterised the FAC-stimulated Fe uptake mechanism. Our studies demonstrate that FAC-stimulated ^{59}Fe uptake from ^{59}Fe-Tf increased biphasically as a function of Tf concentration up to 1 mg/ml ([Fe] = 25 μM). In contrast, internalised ^{59}Fe uptake from ^{59}Fe-citrate became saturated at an Fe concentration of 2.5 μM. Further studies demonstrated that incubation of cells with FAC at 4°C instead of 37°C prevented the stimulation of ^{59}Fe uptake observed, suggesting that an active process was involved. In contrast to previous studies which showed that incubation with FAC stimulates oxidoreductase activity, no change was observed in the ability of cells to reduce the oxidoreductase substrate ferricyanide. These studies suggest that the increase in Fe uptake after preincuba-

tion with FAC was not due to enhancement of oxidoreductase activity. Preincubation of cells with a range of metal ions indicated that Fe uptake from Tf was not only stimulated by FAC, but also by other Fe salts and to a lesser extent Ga. In contrast to Fe, other biologically significant metal ions (e.g. Cu(II) and Zn(II)) did not stimulate Fe uptake from Tf, and actually depressed this process.

2. Materials and methods

2.1. Materials

Iron-59 (as ferric chloride in 0.1 M HCl) was purchased from Dupont, NEN products (Boston, MA, USA). Eagle's modified minimum essential medium (MEM), Hanks' balanced salt solution (BSS), and penicillin-streptomycin were obtained from Gibco (Grand Island, NY, USA). Human Tf was obtained from Sigma (St. Louis, MO, USA). Pronase was purchased from Boehringer-Mannheim (Mannheim, Germany). Non-essential amino acids (100× concentrate) and fetal calf serum (FCS) were obtained from ICN (CA, USA). Fungizone (amphotericin B) was obtained from Squibb (Montreal, Canada). Ferric ammonium citrate (17.5% Fe) was obtained from Aldrich (Sydney, Australia). All other chemicals were of analytical reagent quality.

2.2. Cells

The SK-Mel-28 human malignant melanoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The SK-Mel-28 line was used as the effect of FAC on Fe uptake has previously been examined using these cells and their Fe metabolism is well characterised [10,11,21,22]. Cells were subcultured and used for experiments as described by Richardson and Baker [10]. Procedures used to check cell viability and dedifferentiation were the same as reported previously [10].

2.3. Protein purification and labelling

Human apoTf was prepared and labelled with ^{59}Fe to produce diferric Tf, as described [10]. Unbound ^{59}Fe was removed by exhaustive vacuum dialysis against NaCl (0.15 M) adjusted to pH 7.4 with 1.4% sodium bicarbonate. The saturation of Tf with Fe was monitored by UV-Vis spectrophotometry with the absorbance at 280 nm (protein) being compared with that at 465 nm (Fe-binding site). In all studies, fully saturated diferric Tf was used.

2.4. Experimental procedure: uptake of iron and transferrin after exposure to ferric ammonium citrate

2.4.1. Preincubation procedure

After 24 h on the petri dish, near confluent monolayers of cells were washed to remove bovine Tf from FCS using three separate 30 min incubations in MEM at 37°C. The cells were then preincubated with MEM (control) or MEM containing FAC (25 μg/ml; [Fe] = 4 μg/ml) for 3 h at 37°C. We have used FAC in these studies as Fe-citrate is found in the serum of patients with Fe-overload disease [20]. Hence, this complex has pathophysiological relevance. Also, FAC has been shown to efficiently supply Fe to physiological pools, resulting in downregulation of the TfR [21] and donation of Fe to ferritin [24]. As detailed in our previous studies [22,23], the low concentrations of FAC used in the present studies had no effect on cellular viability as judged by phase-contrast microscopy, Trypan blue staining, and the adherence of cells to the culture substratum. Further, the addition of FAC to the culture medium had no effect on its pH. In some experiments the ability of FAC to increase ^{59}Fe uptake from ^{59}Fe-Tf was compared to a variety of metal ions added to the control medium. For all studies, FAC and the metal ion solutions were prepared in MEM immediately prior to the experiment.

2.4.2. Labelling procedure

After the preincubation procedure, the medium was removed and then replaced with medium containing ^{59}Fe-Tf ([Tf] = 0.002–1 mg/ml; [Fe] = 0.05–25 μM) or ^{59}Fe-citrate (1:100; [Fe] = 0.05–5 μM) which was then incubated with the cells for 3 h at 37°C [22,25]. Previous studies investigating low M_r Fe uptake have used nitrilotriacetic acid (NTA) to chelate ^{59}Fe [26]. However, NTA is not physiologically relevant, and in the present investigation ^{59}Fe-

citrate was used, as this is the prevailing form of non-Tf-bound Fe in the serum of Fe-overloaded patients [20]. In addition, previous studies in melanoma cells have demonstrated that ^{59}Fe donated to the cell from citrate-^{59}Fe complexes is efficiently internalised and incorporated into ferritin and other subcellular compartments [25].

After incubation with ^{59}Fe-Tf or ^{59}Fe-citrate, the petri dishes were placed on ice, the medium removed, and the cell monolayer washed four times with ice-cold BSS. Pronase (1 mg/ml) was then incubated with the cells for 30 min at 4°C to separate internalised (pronase-resistant) from membrane-bound (pronase-sensitive) ^{59}Fe using established techniques [21,27]. Control experiments in our previous studies have shown that this technique is appropriate to measure internalised and membrane-bound ^{59}Fe [10,25,28]. Radioactivity was measured using a γ-scintillation counter (1282 Compugamma; LKB Wallace, Turku, Finland).

Results were calculated as moles of Fe or Tf per gram of protein (gPR) or as a percentage of the control and are expressed as mean or mean ± S.D. Each determination was derived from one confluent petri dish containing 5×10^6 cells, and this was equivalent to approx. 0.45 mg of protein via the bicinchoninic acid protein assay procedure [10].

2.5. Oxidoreductase activity assay

To determine if preincubation of cells with FAC could increase oxidoreductase activity, and therefore Fe uptake, we determined the reduction of the classical oxidoreductase substrate ferricyanide [13]. In this study we examined the reduction of ferricyanide to ferrocyanide using procedures described by Avron and Shavit [29] and Inman et al. [30]. The cells were grown to near confluence on 25 cm^2 culture flasks and washed using three separate 30 min incubations at 37°C. They were then preincubated with MEM (control) or MEM containing FAC (25 μg/ml) for 3 h at 37°C. Cells were then incubated with ferricyanide (50 μM) in phenol red free BSS for 2 or 3 h at either 4°C or 37°C. The measurements at 4°C were used to assess non-specific ferricyanide reductase activity [30]. After the incubation with ferricyanide, 1 ml of the medium overlying the cells was sampled and then centrifuged at 14 000 rpm for 1 min. A 700 μl aliquot of the supernatant was transferred to a cuvette and the following reagents then sequentially added: 100 μl of sodium acetate (3 M, pH 6.4), 100 μl of citric acid (0.2 M), 50 μl of bathophenanthroline disulphonate (3.34 mg/ml), and 50 μl of $FeCl_3$ (3.3 mM freshly prepared in 0.1 M acetic acid) [30]. The solutions were then well mixed and the absorbance measured at 535 nm after 1 h to allow for colour development [30]. All spectrophotometric readings were performed using a Beckman DU 640 UV-Vis spectrophotometer (Beckman Instruments, CA, USA).

2.6. Statistics

Experimental data were compared using Student's *t*-test. Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. The effect of diferric transferrin or citrate concentration on FAC-activated iron uptake

After preincubation of SK-Mel-28 melanoma cells with FAC (25 μg/ml) for 3 h at 37°C, internalised ^{59}Fe uptake from ^{59}Fe-Tf increased biphasically as a function of Tf concentration up to 1 mg/ml ([Fe] = 25 μM) and did not plateau (Fig. 1A). Iron uptake into the pronase-sensitive membrane compartment also increased as a function of Tf concentration (Fig. 1B). The effect of FAC at increasing membrane-bound ^{59}Fe uptake was observed at all Tf concentrations (Fig. 1B). This is in contrast to internalised ^{59}Fe uptake, where FAC only increased ^{59}Fe uptake at Tf concentrations of 0.1 mg/ml or greater (Fig. 1A). These observations may indicate the ^{59}Fe labelling of a membrane-bound molecule involved in FAC-activated Fe transport prior to the increase observed in internalised ^{59}Fe uptake. As described previously, preincubation of cells with citrate alone did not stimulate ^{59}Fe uptake from ^{59}Fe-Tf [22].

In contrast to the FAC-induced increase in internalised ^{59}Fe uptake from ^{59}Fe-Tf (Fig. 1A), internalised ^{59}Fe uptake from ^{59}Fe-citrate plateaued at an Fe concentration of approx. 2.5 μM (Fig. 2A). Furthermore, the extent of the increase in ^{59}Fe uptake after

preincubation with FAC was far greater in cells labelled with ^{59}Fe-citrate compared to those labelled with ^{59}Fe-Tf. For example, at an Fe concentration of 5 μM, ^{59}Fe-citrate uptake was over 20-fold that obtained when cells were preincubated with MEM (Fig. 2A). For comparison, when cells were labelled with ^{59}Fe-Tf at the highest Fe concentration of 25 μM, FAC-activated ^{59}Fe uptake from Tf increased 7-fold (Fig. 1A). Similarly to internalised

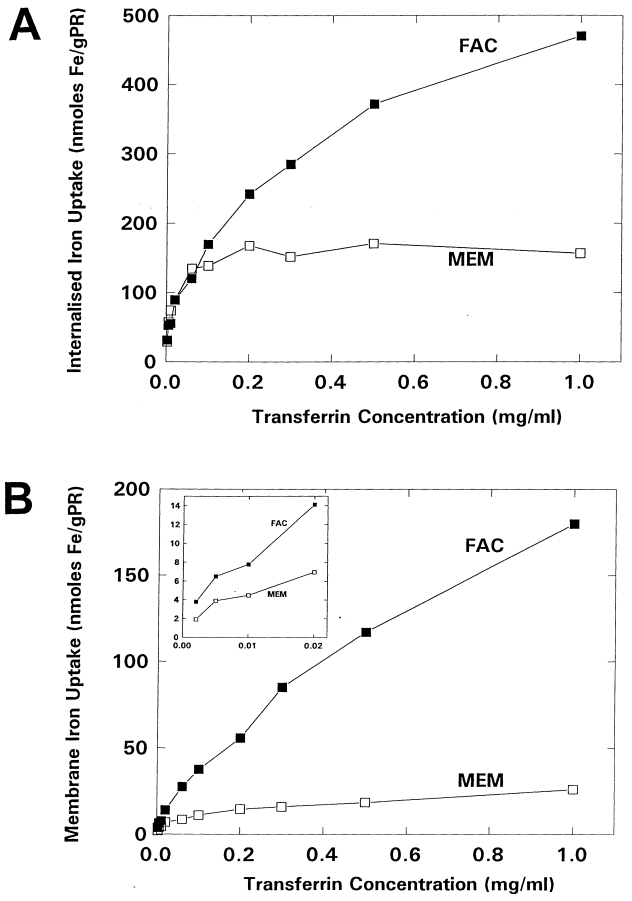

Fig. 1. The effect of ^{59}Fe-transferrin concentration on FAC-activated iron uptake by SK-Mel-28 cells. The cells were washed and subsequently preincubated for 3 h at 37°C with FAC (25 μg/ml) or MEM only and then labelled with ^{59}Fe-Tf (0.002–1 mg/ml; [Fe] = 0.05–25 μM) for 3 h at 37°C. The cells were then washed four times with ice-cold BSS and incubated for 30 min at 4°C with the protease, pronase (1 mg/ml), to separate (A) internalised ^{59}Fe from (B) membrane-bound ^{59}Fe. Inset in B shows membrane-bound ^{59}Fe uptake between ^{59}Fe-Tf concentrations of 0.002 and 0.02 mg/ml ([Fe] = 0.05–0.5 μM). The results illustrated are means of duplicate determinations in a typical experiment of three separate experiments performed.

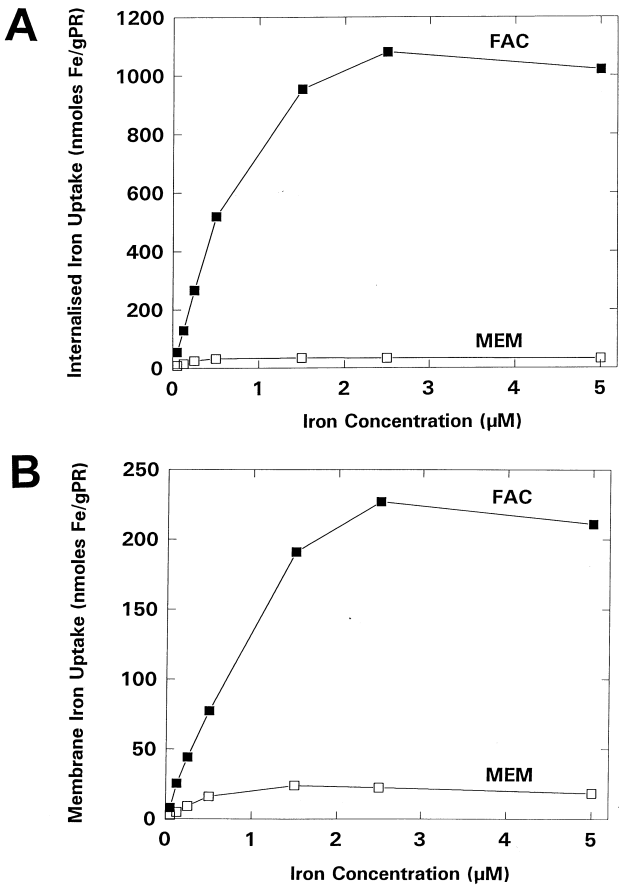

Fig. 2. The effect of ^{59}Fe-citrate concentration on FAC-activated iron uptake by SK-Mel-28 melanoma cells. The cells were washed and subsequently preincubated for 3 h at 37°C with FAC (25 μg/ml) or MEM only and then labelled with ^{59}Fe-citrate ([Fe] = 0.05–5 μM) for 3 h at 37°C. The cells were then washed four times with ice-cold BSS and incubated for 30 min at 4°C with the protease, pronase (1 mg/ml), to separate (A) internalised ^{59}Fe from (B) membrane-bound ^{59}Fe. The results illustrated are means of duplicate determinations in a typical experiment of three separate experiments performed.

^{59}Fe uptake from ^{59}Fe-citrate (Fig. 2A), membrane ^{59}Fe uptake from this complex also plateaued at approx. 2.5 μM (Fig. 2B), suggesting this process was saturable.

3.2. *Effect of preincubation and labelling temperature on FAC-activated iron uptake from transferrin*

Further experiments examined whether the FAC-activated Fe uptake mechanism was temperature-dependent (Fig. 3). Previous studies have clearly demonstrated that Fe uptake from Tf via RME is a tem-

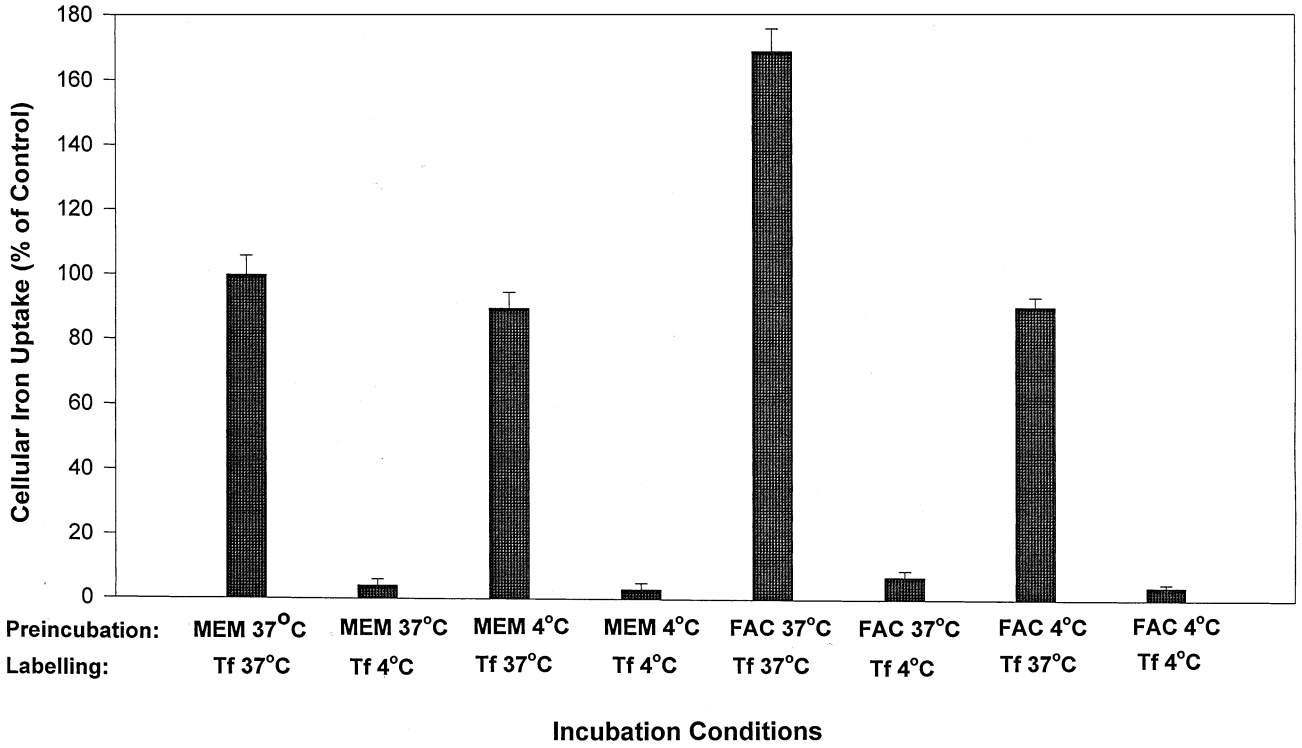

Fig. 3. The effect of preincubation or labelling temperature on iron uptake from transferrin by the FAC-activated iron uptake mechanism by SK-Mel-28 melanoma cells. Cells were washed and subsequently preincubated with FAC (25 μg/ml) or control medium (MEM) alone for 3 h at 4°C or 37°C and then labelled at 4°C or 37°C for 3 h with ^{59}Fe-transferrin (0.1 mg/ml; [Fe] = 2.5 μM). The cells were then washed and internalised ^{59}Fe uptake determined using a 30 min incubation at 4°C with the protease pronase (1 mg/ml). The results shown are mean ± S.D. of three determinations in a typical experiment of three separate experiments performed.

perature- and energy-dependent process [1]. However, nothing is known concerning FAC-activated Fe uptake from Tf. In the present experiments, cells were preincubated with MEM or FAC at 4°C or 37°C and then labelled with ^{59}Fe-Tf at 4°C or 37°C (Fig. 3). Preincubation of cells with FAC at 4°C followed by labelling with ^{59}Fe-Tf at 37°C totally prevented the stimulation of ^{59}Fe uptake observed when cells were preincubated and labelled at 37°C (Fig. 3). In addition, when cells were preincubated with FAC at 37°C and then labelled with ^{59}Fe-Tf at 4°C, again no stimulation of ^{59}Fe uptake from ^{59}Fe-Tf was found (Fig. 3). These results suggested that the FAC-stimulated Fe uptake process was an active temperature-dependent mechanism.

Studies were initiated to examine the effect of a range of metabolic inhibitors on FAC-activated ^{59}Fe uptake from ^{59}Fe-Tf (data not shown). However, the results were difficult to interpret because when inhibitors (e.g. 2,4-dinitrophenol) were added to FAC, they may not only be acting to reduce ATP levels, but could also be directly reacting with FAC to influence the result.

3.3. Is FAC-activated iron uptake mediated by stimulation of a cell surface oxidoreductase?

Previous studies by others suggested that FAC could stimulate cell surface oxidoreductase activity [31,32]. Such a mechanism could possibly explain the ability of FAC to increase ^{59}Fe uptake from ^{59}Fe-Tf. To test this hypothesis, cells were preincubated with FAC for 3 h at 37°C and oxidoreductase activity measured by assessing the reduction of the standard oxidoreductase substrate ferricyanide at 4°C or 37°C for 2 or 3 h [13] (Fig. 4). The measurements at 4°C were relevant controls to determine the amount of non-specific ferricyanide reduction that is not due to active cellular oxidoreductase activity [30]. Reduction of ferricyanide at 37°C was always greater

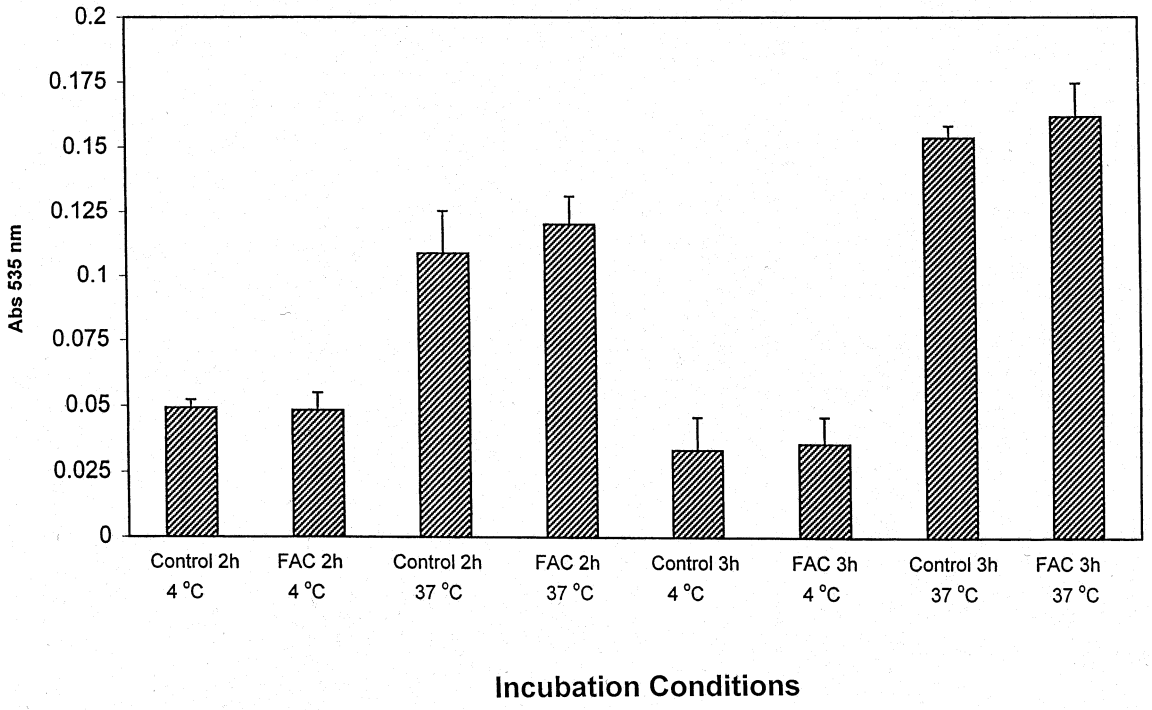

Fig. 4. The effect of preincubation with FAC on the ability of cells to reduce the oxidoreductase substrate ferricyanide. The SK-Mel-28 melanoma cells were washed and subsequently preincubated for 3 h with control medium or FAC (25 μg/ml) at 37°C. The cells were then washed, and their ability to reduce ferricyanide examined by incubation with this compound at 4°C or 37°C for 2 or 3 h (for details see Section 2). The results shown are mean ± S.D. of three determinations in a typical experiment of three separate experiments performed.

than that found at 4°C (Fig. 4). However, compared to the relevant control, preincubation with FAC had no significant effect ($P > 0.05$) on the reduction of ferricyanide after 2 or 3 h (Fig. 4). These data suggest that FAC was not stimulating oxidoreductase activity and increasing ^{59}Fe uptake from ^{59}Fe-Tf by this mechanism. However, from the current data we cannot exclude that the reductase activity observed plays some role in Fe transport.

3.4. The effect on iron uptake from transferrin of preincubation of cells with a range of metal ions

To further examine the mechanism of the FAC-activated Fe uptake, cells were incubated with a range of metal salts (25–200 μM) including ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$), ferrous ammonium sulphate (FAS), gallium nitrate ($Ga(NO_3)_3$), copper chloride ($CuCl_2$), zinc chloride ($ZnCl_2$), or cobalt chloride ($CoCl_2$), and the results compared to FAC (Fig. 5). At the concentrations of metal ions tested, no precipitation was evident during the experiment. Previous studies have used metal ion concentrations from 0.5 mM up to 1 mM to examine their effect on metal transport [26,33]. However, in our hands, concentrations greater than 200 μM were found to be cytotoxic and/or resulted in precipitation, and so were not used. In the case of the Fe salts, the high concentrations of natural chelators within MEM (e.g. amino acids, phosphate etc.) was sufficient to prevent precipitation of high M_r Fe hydroxide species.

The three most effective metal ions at increasing ^{59}Fe uptake from ^{59}Fe-Tf were (in descending order) $FeCl_3$, $FeSO_4$, and FAS (Fig. 5). At an Fe concentration of 200 μM, each of these metal salts increased ^{59}Fe uptake from ^{59}Fe-Tf by approx. 250%, 200%, and 175% of the control value respectively (Fig. 5). In contrast, preincubation with FAC resulted in a biphasic response resulting in an initial increase in ^{59}Fe uptake to over 150% of the control at 50 μM, followed by a subsequent decrease in ^{59}Fe uptake as

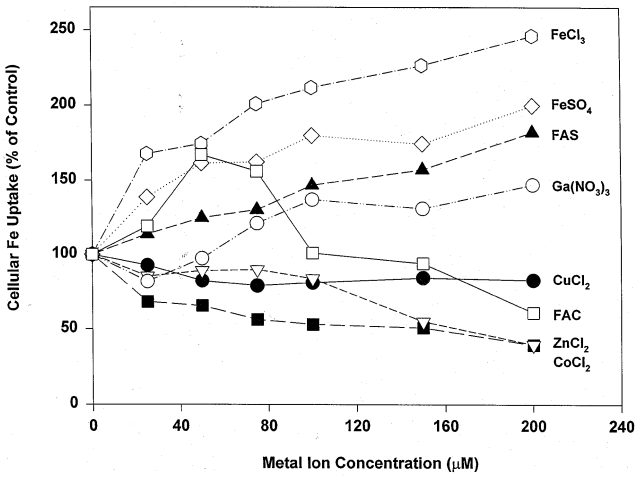

Fig. 5. The effect of preincubating cells with a variety of metal ions on iron uptake from ^{59}Fe-transferrin by SK-Mel-28 melanoma cells. The cells were washed and subsequently preincubated for 3 h at 37°C with metal ions (25–200 μM) or control medium only and then labelled with ^{59}Fe-transferrin (0.1 mg/ml; [Fe] = 2.5 μM) for 3 h at 37°C. The cells were then washed four times with ice-cold BSS and incubated for 30 min at 4°C with the protease, pronase (1 mg/ml), to separate internalised from membrane-bound ^{59}Fe. The results illustrated are means of duplicate determinations in a typical experiment of three separate experiments performed.

the concentration increased (Fig. 5). The reason for this biphasic response remains unclear at present, but was not due to toxicity, as the cells remained attached to the substratum and were greater than 98% viable. The only other metal ion that increased ^{59}Fe uptake from ^{59}Fe-Tf was $Ga(NO_3)_3$. However, the effect of this metal ion was less marked at stimulating ^{59}Fe uptake than the Fe salts, increasing ^{59}Fe uptake to less than 150% of the control value. The other metal salts decreased ^{59}Fe uptake from ^{59}Fe-Tf as the concentration of the metal ion increased, the most effective being $ZnCl_2$ and $CoCl_2$ (Fig. 5).

4. Discussion

In the present study we have further examined the mechanism of the FAC-stimulated Fe uptake process from Tf and Fe-citrate. To date, a variety of studies have examined the effect of FAC and metal ions on the uptake of small M_r complexes [19,33–37]. In contrast, none of these investigations have examined the mechanism involved in FAC-activated Fe uptake from Tf. In the present study we demonstrate that a variety of Fe salts (ferric chloride, ferrous ammonium sulphate and ferrous sulphate), and to a lesser extent $Ga(NO_3)_3$, are effective at increasing Fe uptake from Tf. In contrast, other metal ions including Zn(II), Co(II), or Cu(II) inhibit Fe uptake. The effect of these Fe and Ga salts at increasing ^{59}Fe uptake from ^{59}Fe-Tf has not been reported previously, although there have been reports indicating that Ga and Fe salts can stimulate Fe uptake from Fe-NTA and other low M_r complexes [19,33,35].

Our previous studies showed that stimulation of Fe uptake from either Tf or Fe-citrate was linked to the ability of Fe to generate free radicals via the Haber-Weiss reaction [22]. Interestingly, Ga(III) cannot redox cycle between the +2 and +3 redox states [39] to generate free radicals. Yet, this metal can stimulate Fe uptake from Tf, albeit less efficiently than equimolar concentrations of Fe (Fig. 5). These results may indicate that the Fe uptake process stimulated by Fe and Ga is via separate transport mechanisms. Alternatively, the extent of induced transport may be dependent on a number of factors. For instance, while free radical production appeared important in terms of activating the Fe uptake mechanism [22], it cannot be excluded that the presence of the Fe atom itself or a metal ion with atomic similarities to Fe may be vital for the full response. Binding of the metal ion to an appropriate receptor site may also be an essential component for activation. Considering this, it should be noted that an increase in membrane Fe binding was observed after incubation with FAC (Fig. 1B), suggesting the involvement of a membrane Fe-binding molecule. While Ga(III) does not produce free radicals in solution, this cation is similar to Fe(III), and can interact with Fe-binding sites in a variety of molecules [40,41]. The fact that Ga(III) was less effective than any of the Fe salts at activating Fe transport lends some support to a mechanism requiring several triggering factors. This hypothesis is further supported by the observation that Cu(II) which can redox cycle had no stimulatory effect on Fe uptake from Tf.

The FAC-activated Fe uptake mechanism has been observed in a wide variety of cell types apart from SK-Mel-28 melanoma cells, including primary cultures of hepatocytes, myocardial cells, and fibro-

blasts, as well as many cell lines including: HeLa cells, HL-60 cells, HepG2 hepatoma cells, Chinese hamster ovary cells, a human embryonic kidney cell line, and L-cells [19,22,34,36–38]. In the present study, we have used the SK-Mel-28 melanoma cell line as a convenient model as our previous studies have examined the effect of FAC on these cells [21,22,42], and its Fe metabolism is well characterised [10,11,25]. The wide distribution of this Fe uptake process in a variety of cell types may indicate this mechanism has general biological relevance rather than being a process specific for a highly specialised cell type. Such a function could include a cellular defense mechanism against redox-active Fe complexes as suggested previously [22]. In this way, toxic extracellular Fe would be internalised and incorporated into a non-toxic form within ferritin.

Experiments in the present study clearly demonstrate the difference between FAC-activated ^{59}Fe uptake from ^{59}Fe-Tf and ^{59}Fe-citrate by melanoma cells. In the case of FAC-activated Fe uptake from ^{59}Fe-citrate, the process is saturable as a function of Fe concentration, and leads to a 20-fold increase in ^{59}Fe uptake. In contrast, FAC-activated ^{59}Fe uptake from ^{59}Fe-Tf is only clearly observed when the Tf concentration is 0.1 mg/ml or above, and does not saturate even at a Tf concentration of 1 mg/ml ([Fe] = 25 μM; Fig. 1A). This contrasts with internalised ^{59}Fe uptake from ^{59}Fe-citrate which saturates at an Fe concentration of 2.5 μM. Furthermore, the extent of stimulation of ^{59}Fe uptake from ^{59}Fe-Tf after incubation with FAC was far less than that seen with ^{59}Fe-citrate (cf. Figs. 1A and 2A). These studies suggest that the ^{59}Fe-citrate complex may interact directly with the transport mechanism, while its effect on Tf appears indirect. In previous studies we showed that non-specific binding of Tf to the cell surface appeared to be essential for FAC-activated Fe transport [22]. Further, this mechanism was shown to be independent of the high affinity TfR [22].

Our present study demonstrates that the effect of FAC at increasing ^{59}Fe uptake was temperature-dependent (Fig. 3). These experiments showed that active processes were involved in both the activation event initiated by the preincubation with FAC, and also the subsequent increase in ^{59}Fe transport during the labelling period. Critically, one could suggest that FAC-activated ^{59}Fe uptake at 37°C may be explained by the ability of FAC to increase cell permeability via membrane damage, allowing passive influx of the ^{59}Fe label. However, this is not the case, because preincubating cells with FAC at 37°C followed by labelling at 4°C resulted in no enhancement of ^{59}Fe transport (Fig. 3). Furthermore, preincubating cells with FAC at 4°C followed by incubation with Tf at 37°C did not stimulate ^{59}Fe uptake (Fig. 3). These latter results suggest that cellular metabolism was essential to detect and respond to the increased levels of extracellular Fe.

Studies examining the reduction of ferricyanide suggested that preincubation with FAC did not enhance oxidoreductase activity or stimulate Fe uptake from Tf by this mechanism. These data agree with our previous results showing that the oxidoreductase inhibitor, amiloride, had no effect on FAC-activated Fe uptake [22]. The role of an oxidoreductase in Fe uptake from Tf [43] is highly controversial, and has been challenged on both technical and theoretical grounds [44]. However, there have been reports suggesting that reduction of the FAC-Fe(III) complex to the Fe(II) state may occur, resulting in dissociation of the complex and the independent uptake of citrate and Fe [35]. Our present studies indicate that ferricyanide reductase activity is present on cells, and that it does not increase after incubation with FAC. However, we cannot exclude that the reductase activity observed could play some role in Fe uptake.

The finding that Ga or a number of Fe salts added to MEM stimulates Fe uptake from Tf suggests that the initial activation of this process is not dependent on just the ferric citrate complexes found in FAC. It should be mentioned that the addition of Fe or Ga salts to medium will result in the formation of a wide variety of weak complexes with medium constituents such as amino acids and phosphate. A critical characteristic for inducing Fe transport appears to be the formation of relatively weak complexes that are labile. Indeed, our previous studies showed that an excess of the strong Fe chelator, desferrioxamine, when added to FAC prevented its stimulatory effect on Fe transport [22].

While the molecular basis for FAC-activated Fe uptake remains unknown, it is of interest that an Fe-activated, P-type ATPase involved in Fe transport has recently been identified [45]. In this latter

study, ATPase-mediated Fe transport was induced 2-fold within the first 1–2 h following incubation with $FeSO_4$, followed by a 5-fold induction after an 18 h incubation [45]. The ATPase was suggested to be found in microsomes and endosomes, and may be involved in transporting Fe released from haem by haem oxygenase-1 [45]. Further work is required to determine if this molecule could also be expressed on the cell surface and have some role in FAC-activated Fe uptake.

Regarding the role of ATPases in metal ion transport, it is of interest to note that another P-type ATPase known as the Menkes protein is involved in Cu transport and becomes redistributed from the Golgi to the plasma membrane upon exposure to extracellular Cu [46]. Considering that Cu and Fe can bind to similar ligating sites [39], it could be suggested that the Menkes protein is involved in FAC-activated Fe uptake. However, the fact that incubation with Cu did not result in enhanced Fe uptake from Tf (Fig. 5) argues against this proposal. In previous studies, Kaplan and colleagues [36] suggested that the Fe transporter involved in FAC-activated Fe uptake could be due to the movement of transporters from an internal pool to the cell surface. Further studies examining the redistribution of the Fe-activated P-type ATPase or other Fe transporters (e.g. Nramp2 [3–5] and the stimulator of iron transport [47]) after exposure to Fe salts appear important in terms of understanding the molecular basis of FAC-activated Fe uptake.

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