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Monocyte–macrophage ferric reductase activity is inhibited by iron and stimulated by cellular differentiation

Jason PARTRIDGE*, Daniel F. WALLACE*, Kishor B. RAJA†, James S. DOOLEY* and Ann P. WALKER*¹

*Department of Medicine, Royal Free and University College Medical School, Royal Free Campus, Rowland Hill Street, London NW3 2PF, U.K., and

†Department of Clinical Biochemistry, King's College School of Medicine & Dentistry, Bessemer Road, London SE5 9PJ, U.K.

The enzyme ferric reductase catalyses the reduction of Fe(III) as a prerequisite to its transportation across the cell membrane. Duodenal mucosal biopsies from iron overloaded patients with genetic haemochromatosis (GH) have increased ferric reductase activity and iron absorption compared with controls, yet the GH mucosa is iron deficient. A similar GH-related iron deficiency is also seen in macrophages. The aim of this study was to investigate whether macrophage ferric reductase activity is altered in GH, and to determine ferric reductase activity in monocytes and differentiated macrophages. The erythroleukaemic K562 cell line was studied as a clonal reference cell line. The basal K562 ferric reductase activity is characteristic of a membrane bound enzyme, being both temperature and protease sensitive. Ferric reductase activity was also demonstrated in human leucocyte, monocyte and macrophage preparations. Assays of K562 and macrophage

cell supernatants confirmed that the ferric reductase activity was not due to a secreted factor. Assay of ferric reductase in normalized-iron and iron-enriched (100 μ M ferric citrate) conditions showed no significant difference between Cys²⁸²Tyr (Cys²⁸² → Tyr) homozygous GH macrophages and Cys²⁸²-Tyr negative control activities ($P > 0.05$). However, a 900% increase in ferric reductase activity was observed during monocyte to macrophage differentiation ($P < 0.05$), possibly reflecting the co-ordinate up-regulation of iron metabolism in these cells. The demonstration of approx. 25% activity after macrophage differentiation at high free-iron concentrations compared with 'normalized' iron is consistent with repression of human ferric reductase activity by iron. The identification of the human ferric reductase gene and its protein will ultimately provide insight into its regulation and role in mammalian iron metabolism.

INTRODUCTION

Iron is of vital importance in all living organisms, being an essential cofactor of enzymes involved in respiration, cell replication and electron shuttling. The homeostasis of iron is maintained primarily by its absorption in the duodenum and the proximal region of the jejunum [1]. Normally the daily loss of iron (1–2 mg) is matched by absorption from the diet. This fine balance between iron excretion and absorption is lost in genetic haemochromatosis (GH). This autosomal recessive disease leads to increased absorption of iron from the diet despite increasing body iron stores. Iron is deposited in the parenchymal tissue of the liver, pancreas, heart and other tissues. If untreated, the iron overload eventually leads to tissue damage. The prevalence of GH in Europeans is approximately 1 in 300, which is higher than the combined prevalence of cystic fibrosis, phenylketonuria and Duchenne muscular dystrophy [2–4].

Recently a candidate gene for haemochromatosis (*HFE*) was identified [5]. In the United Kingdom, 91% of GH patients were found to be homozygous for a single mutation of *HFE*, Cys²⁸²Tyr (Cys²⁸² → Tyr) [6]. Although the metabolic role of the HFE protein is not fully understood, recent advances support a role in the regulation of iron absorption [7–10].

Abnormalities of iron metabolism in the intestine, liver and macrophages have all been implicated in the pathogenesis of GH [11–13]. In the haemochromatotic macrophage, despite the elevation of serum iron and transferrin saturation, concentrations of iron are minimal until late in the disease [14]. This is in contrast with transfusional siderosis, where there is progressive

accumulation of iron by the macrophage. This difference exists despite similar increases in serum-ferritin levels [15]. Cultured GH monocytes also have elevated levels of ferritin release, and abnormal kinetics of iron release, compared with control monocytes [16,17].

The uptake of 'free' iron provided in the form of ferric citrate by mononuclear cells has been established [18,19]. Both free iron uptake and ferric reductase activity have been shown to be increased in duodenal biopsies from haemochromatosis patients as compared with controls [20,21].

Ferric reductase activity has been described in both prokaryotic and eukaryotic cells, including *Escherichia coli*, *Saccharomyces cerevisiae*, mouse, rat, rabbit and human duodenum, rat liver endosomes, rabbit reticulocytes and various cultured cell lines [21–24]. Its conservation across species indicates a functional role. The *S. cerevisiae* gene for ferric reductase (*FRE1*) has been cloned and sequenced [22,23]. The ferric iron uptake mechanism in the yeast, *S. cerevisiae*, utilizes the ferric reductase enzyme to reduce Fe(III) before absorption. The ferrous Fe(II) iron is transported through the membrane, and is thought to be re-oxidized back to Fe(III) as it passes through the membrane. The human homologue of *FRE1* has yet to be cloned. However, partial purification of human ferric reductase from both human Hutu 80 duodenal adenocarcinoma cells and human duodenal microvillus membranes has shown that the activity is NADH-dependent and the protein is membrane bound [24].

We demonstrate ferric reductase activity in the K562 human erythroleukaemic cell line and primary cultures of human leucocytes, monocytes and macrophages. We report ferric

Abbreviation used: GH, genetic haemochromatosis.

¹ To whom correspondence should be addressed (e-mail a_walker@rfsm.ac.uk).

reductase activity in cells from GH patients compared with controls, under conditions of high free iron and during monocyte-macrophage differentiation.

MATERIALS AND METHODS

Patients

Ten male patients were investigated: five GH and five control patients with no family history of iron disorders. The diagnosis of GH was made from clinical and biochemical data and confirmed by a hepatic iron index > 1.9 and/or removal of > 5 g of iron during initial phlebotomies [3,6,25]. The groups were age matched: GH, 49 ± 17.4 years; control, 55 ± 5.3 years, (mean ± S.D.).

Mutation analysis

The Cys²⁸²Tyr and His⁶³Asp mutations of the haemochromatosis gene, *HFE*, were determined by PCR and restriction enzyme digestion [6]. All haemochromatosis patients studied were homozygous for the Cys²⁸²Tyr mutation and negative for His⁶³Asp. Two controls carried a single copy of His⁶³Asp; all controls were negative for Cys²⁸²Tyr.

Cell culture

White blood cells were extracted from peripheral blood using Lymphoprep[®] (Nycomed). Monocytes were enriched by their adherence to plastic. Adherent monocytes were cultured for 14 days for *in vitro* differentiation to macrophages [26]. Cell numbers were determined using a haemocytometer and by fluorescence activated cell sorter analysis (Coulter EpicXL). Cell viability was confirmed by Trypan Blue exclusion (> 95%). The purity of the primary cell cultures was assessed by morphological criteria (Leishmanns stain) and CD14 fluorescence-activated cell sorter analysis; typical purities were 80% for monocytes and 95% for macrophages. The clonal erythroleukaemic cell line K562 was included in all experiments as an internal control. Cells were cultured at 37 °C either in conditions of 'normalized' iron (24 μM iron, transferrin saturation 30%), or in normalized medium supplemented with 100 μM ferric citrate, to reflect the elevated *in vivo* concentrations of serum iron and free iron of a heavily iron-loaded haemochromatotic [27,28]. The 100 μM ferric citrate solution was freshly prepared as a mixture containing 100 μM ferric chloride and 150 μM sodium citrate, as described previously [29]. Cell cultures supplemented with ferric citrate were protected from light and fresh medium was substituted after 7 days. In addition to the protection from light and chelation by citrate, stability of Fe(III) was also conferred by the RPMI 1640 culture medium itself, which contains many other chelating agents at high concentrations. These include amino acids, sugars, vitamins, bicarbonate and phosphate, which also prevent the hydrolytic polymerization of iron [30]. The amino acids and glucose alone provide an additional 150-fold molar excess of chelating agent over iron [30].

Ferric reductase assay

Cells were harvested, rinsed, then incubated at a concentration of 2 × 10⁶ cells/ml in 200 μl of oxygenated physiological buffer (125 mM NaCl/3.5 mM KCl/1 mM CaCl₂/10 mM MgSO₄/10 mM D-glucose in 16 mM Hepes/NaOH, pH 7.4) in 96-well tissue culture plates at 37 °C. The reaction was started by the addition of 100 μM Fe(III), as a ferric chelate of nitrilotriacetate in a 1:2 ratio (Fe:nitrilotriacetate), and ferrozine (1 mM). The reaction was performed at 37 °C. The rate of reaction was

determined spectrophotometrically at 562 nm by measuring the reduction of the yellow Fe(III) to the purple coloured stable Fe(II)-ferrozine complex [21].

RESULTS

Preliminary experiments investigated the characteristics of the ferric reductase activity of the clonal control K562 erythroleukaemic cell line. In each experiment the rate of ferric reductase activity was determined by linear regression analysis of at least three time courses; each individual time point was performed in duplicate. All time courses were linear for at least 60 min. After mild trypsinization, ferric reductase activity was decreased to 38.7% of the control (Table 1). Heating the cells to 65 °C for 5 min prior to assay decreased activity to 26.7% of the control. When the assay was performed at 10 °C, 17% of the control activity was observed. These observations were consistent with the assay of a membrane bound protein. When K562 and macrophage cell-free supernatants were taken for determining reductase activity at the end of the incubation period, only background levels of reductase activity were detected (Table 1). This indicated that the activity was not due to a soluble, released

Table 1 Human ferric reductase activity has the characteristics of a membrane bound enzyme

The cells were subjected to various treatments and then ferric reductase activity was measured and expressed as a percentage of K562 control activity at 37 °C (mean ± S.D.; n = 3). † The ferric reductase activity for the control K562 cells was 6.93 nmol of Fe · min⁻¹ · (10⁹ cells)⁻¹, where 1 μM ferric iron gave an absorbance of approx. 0.03 at 562 nm. ‡, The reaction was performed in the absence of ferrozine, cells were removed by centrifugation, and the activity in the supernatant was determined after the addition of ferrozine. Activities are compared with the control; **P < 0.01 (Student's *t*-test).

Cell type	Treatment	Ferric reductase activity (%)†
K562	0.1 mg/ml trypsin, 37 °C, 60 min	38.7 ± 1.5**
K562	65 °C, 5 min	26.7 ± 1.5**
K562	Assay performed at 10 °C	17.0 ± 1.7**
K562	Supernatant activity‡	0.33 ± 0.58**
Macrophage	Supernatant activity‡	0.02 ± 0.02**

Table 2 Ferric reductase activities

Activities are shown for lymphocytes, monocytes and macrophages from classical Cys²⁸²Tyr homozygous GH patients and Cys²⁸²Tyr negative control patients. Statistical probabilities were assessed using the Student's *t*-test. There was a significant increase in activity with differentiation of the monocytes to macrophages, for both GH (*) and control (†) preparations (P < 0.05 in both cases). N, normalized-iron culture medium (see the Materials and methods section); 100 μM, iron loaded (additional 100 μM ferric citrate in normalized-iron medium).

Cell type	Fe	Ferric reductase activity [nmol of Fe · min ⁻¹ · (10 ⁹ cells) ⁻¹]		
		Mean ± S.D.	n	P
Lymphocytes	GH	N	4.37 ± 3.13	3 } > 0.05
	Control	N	3.98 ± 2.51	
Monocytes	GH	N	4.38 ± 1.71*	3 } > 0.05
	Control	N	3.75 ± 0.71†	
Macrophages	GH	N	43.3 ± 5.13*	4 } > 0.05
	Control	N	31.3 ± 8.67†	
Cell line	K562	N	6.93 ± 0.88	3 } > 0.05
	K562	100 μM	7.87 ± 0.77	
Macrophages	GH	100 μM	10.0 ± 4.87	5 } > 0.05
	Control	100 μM	9.2 ± 9.60	

factor. These observations indicate that ferric reductase is a membrane bound enzyme.

Investigation of primary cultures of lymphocytes, monocytes and macrophages demonstrated ferric reductase activity in these blood-derived cells (Table 2). No significant difference ($P < 0.05$) was observed between GH (Cys²⁸²Tyr homozygous) and control (Cys²⁸²Tyr negative) in either lymphocyte, monocyte or macrophage preparations. However, ferric reductase activity of macrophages was approx. 900% of the monocyte activity, for both GH and control preparations, when cultured in normalized iron.

When K562 cells were cultured in normal or high iron (100 μ M ferric citrate) concentrations, no difference in ferric reductase activity was observed ($P > 0.05$; Table 2)

When macrophages were cultured in media containing 100 μ M ferric citrate for 2 weeks, no significant difference ($P > 0.05$) was observed between GH and the control. However, the ferric reductase activity of both GH and control macrophages cultured under these conditions was approx. 25% of the activity when grown at normalized iron concentrations ($P < 0.05$; Table 2).

DISCUSSION

The ferric reductase activity of human K562 cells was demonstrated to have the characteristics of a membrane bound enzyme and was not attributable to the secretion of reducing factors. There was no significant difference in the reductase activity between GH and controls for leucocyte, monocyte or macrophage preparations. This was observed under both normalized iron conditions and in the presence of 100 μ M ferric citrate. Ferric reductase activity of differentiated macrophages was approx. 900% that of monocyte activity ($P < 0.05$). This increase most likely reflects the co-ordinated up-regulation of the proteins of iron metabolism during the transition into macrophages [31]. Macrophage ferric reductase activity was decreased when cultured in 100 μ M ferric citrate compared with that in normalized iron. From these results it would appear that macrophage ferric reductase activity may be determined by external free-iron concentrations during macrophage differentiation. Macrophage ferric reductase activity was down-regulated by increased free-iron concentration.

This control of ferric reductase activity could contribute to the low levels of iron observed in the haemochromatotic macrophage. As haemochromatotic macrophages differentiate in high free-iron conditions *in vivo*, their ferric reductase activities may be decreased according to the amount of free iron present. Future studies could address this issue by isolating differentiated macrophages from GH and control human subjects, and measuring the activity of the *in vivo* differentiated cells. The clonal reference cell line K562 did not show modulation of ferric reductase activity by iron. The K562 cell line originates from an erythroid cell clone derived from a patient with myeloid leukaemia in acute blast crisis [32]. The lack of modulation of K562 ferric reductase activity by iron may reflect either the erythroid lineage or changes in the clonal expansion of this cell line. The identification of the human ferric reductase gene will ultimately establish whether it is transcriptionally repressed by iron, as in *S. cerevisiae*. Future studies will elucidate whether ferric reductase plays a role in the apparent iron deficiency of macrophages in GH.

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