Impact of iron treatment on immune effector function and cellular iron status of circulating monocytes in dialysis patients

Thomas Sonnweber¹, Igor Theurl¹, Markus Seifert¹, Andrea Schroll¹, Susanne Eder², Gert Mayer² and Günter Weiss¹

¹Department of Internal Medicine I, Clinical Immunology and Infectious Diseases and ²Department of Internal Medicine IV, Nephrology and Hypertension, Medical University of Innsbruck, Innsbruck, Austria

Correspondence and offprint requests to: Günter Weiss; E-mail: guenter.weiss@i-med.ac.at

Abstract

Background. Iron is used as an adjunct therapy to treat anaemia in dialysis patients. However, iron may harbour detrimental effects in being a nutrient for invading pathogens or by modulating immune pathways. Thus, we prospectively studied the effects of iron treatment towards monocyte immune function.

Methods. Twenty-four haemodialysis patients in whom iron therapy was withheld for 2 weeks before study entry were randomly assigned to receive either a single parenteral dose of iron sucrose or saline (control), and the effects on iron status and immune function of patients’ monocytes were analysed during follow-up.

Results. At baseline, we found an inverse relationship between serum ferritin levels and the inducibility of patients’ monocytes for interleukin-6 and tumour necrosis factor-alpha formation following ex vivo immune stimulation. However, 48 h after intravenous iron administration, we observed a transient increase of tumour necrosis factor-alpha and interleukin-6 formation by unstimulated monocytes as compared with control subjects. This could be traced back to increased phosphorylation of nuclear factor kappa-B p65 in monocytes following iron treatment, which was more pronounced when pre-treatment serum ferritin levels were low. In parallel, intravenous iron injection resulted in storage of the metal in circulating monocytes as reflected by an increase of intracellular ferritin levels, and the amount of iron retention was positively associated with circulating concentrations of the iron regulatory peptide hepcidin.

Conclusions. Intravenously administered iron is taken up by monocytes and leads to short-term activation of the nuclear factor kappa-B pathway. However, increased circulating ferritin levels are associated with an impaired immune response of monocytes. In addition, circulating hepcidin concentrations may determine the erythropoietic response to iron injection by modifying iron retention within monocytes.

Keywords: end-stage renal disease; innate immune response; iron therapy; monocytes; renal anaemia

Introduction

Anaemia is frequently found in patients with end-stage renal disease (ESRD) [1]. The prevalence of anaemia is directly related to renal dysfunction and progressively increases when the glomerular filtration rate declines [2]. Anaemia is significantly associated with mortality and morbidity in ESRD patients and has been linked to the development of left ventricular hypertrophy and cardiovascular disease [3–6].

The pathophysiology of anaemia in ESRD is multifactorial. Relative deficiency of erythropoietin, a diminished proliferation of erythroid progenitor cells on the basis of accumulation of uraemic toxins, as well as chronic blood loss due to dialysis procedure, and vitamin deficiencies play major roles [1,7,8]. In addition, a chronic inflammatory status due to the dialysis procedure with subsequent activation of immune cells as well as subclinical bacteremia on the basis of repeated shunt punctures is often present in dialysis patients [1]. Chronic inflammation leads to iron retention within monocytes/macrophages with limitation of iron availability for erythropoiesis, cytokine-mediated inhibition of erythroid progenitor cell proliferation and a diminished biological activity of erythropoietin, all of which being established cornerstones in the development of anaemia of chronic disease (ACD) [9,10]. In addition, advanced inflammation is associated with increased mortality and poor response to anaemia treatment [11,12]. Anaemia in ESRD patients is mostly treated with erythropoiesis-stimulating agents (ESA) to compensate for the erythropoietin deficiency [7,10]. However, the response to ESA is sometimes poor due to iron deficiency, mainly as a consequence of chronic blood loss during the dialysis procedure, and/or due to iron retention within monocytes/macrophages [13].

Thus, guidelines recommend regular assessment of iron status in ESRD patients with anaemia [1,14] since ESA treatment by itself can further contribute to iron deficiency by mobilizing the metal for erythropoiesis [7,10].
Iron deficiency in ESRD patients is treated with intravenous iron [15]. However, the monitoring of iron status is difficult in the setting of ESRD and inflammation since the major iron storage protein ferritin is not only induced by iron overload but also by inflammation [16,17]. Thus, guidelines recommend to maintain serum ferritin concentrations at 200 ng/mL or higher [1,14,18]. However, iron overload can harbour detrimental effects by promoting intravascular radical formation, by increasing the availability of the essential nutrient iron for microorganisms and by modulating immune effectors pathways [19,20].

Importantly, so far, hardly any information has been available to what extent intravenously administrated iron may be taken up by circulating monocytes and to what extent this may affect their immune response. These issues were addressed in the current work.

Materials and methods

Patients

A total of 24 haemodialysis patients were included into this study. The study was approved by the local ethical committee at the Medical University of Innsbruck, and written informed consent was obtained in accordance with the Declaration of Helsinki. Among the 24 participating haemodialysis patients, 13 suffered from diabetes mellitus, 3 suffered from IgA nephropathy, 3 presented with hypertensive nephropathies, 1 suffered from Goodpasture’s syndrome and 2 had end-stage renal disease due to unknown aetiology. Serious infectious complications, such as pneumonia and central venous catheter infections, had previously been described in the clinical history of 11 out of the 24 patients. Patients with elevated C-reactive protein (CRP) levels (>10 mg/L) or clinical signs of acute infection during the enrolment period were excluded from the study. Importantly, in none of our patients was a significant increase observed in CRP levels during the study period of 1 week (details not shown).

Patients were randomly assigned to the control (n = 8) or the intervention group (n = 16). All patients were withdrawn from iron therapy for at least 2 weeks before study entry. Mean rhErythropoietin (rhEPO) dosage prior study entry is shown in Table 1. Whereas patients from the intervention group received a single dose of 100-mg intravenous iron sucrose (Vénofér®, Vifor, Neuilly-sur-Seine, France) at Day 0, control individuals were given the same volume of sterile saline, both at the end of haemodialysis. Blood was drawn before iron injection at Day 0 and after 48 h and 1 week, immediately before dialysis. Basic laboratory parameters were determined by routinely used automated laboratory tests. Serum TNF-α and serum IL-6 were measured by ELISA (R&D Systems, Minneapolis, MN, USA). Serum hepcidin-25 was retrospectively determined in a subgroup of patients for whom serum was available as previously described [16,21].

Patients were dialysed three times a week for 3–5 h. Dialysis prescriptions were constant during the study period, and there were no significant differences between the control and intervention group as far as the dialysis procedure is concerned. All patients underwent chronic dialysis for at least 1 year in order to exclude changes due to newly introduced dialysis procedures.

Cell culture and ex vivo experiments

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from whole blood by Ficoll–Paque separation (Pharmacia®, Uppsala, Sweden) as previously described [22]. PBMCs were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (vol/vol) heat-inactivated fetal calf serum (PAA Laboratories Gmbh, Pasching, Austria), 1% penicillin–streptomycin solution and 100 µM 2-mercaptoethanol.
100 U/mL penicillin and 0.1 mg/mL streptomycin, and seeded into a 100-mm dish or six-well plates, respectively. Monocytes were allowed to adhere in a 5% CO₂ incubator at 37°C for 2 h. Non-adherent cells were removed, and monocytes were washed gently with pre-warmed PBS.

Monocytes were stimulated with either 500 ng/mL Escherichia coli lipopolysaccharide (LPS; 055:B5; Sigma, Munich, Germany) or 500 U/mL rh-interferon-gamma (rhIFN-γ Life Technologies, Gaithersburg, MD, USA) or the same volume of RPMI 1640 medium for controls and incubated at the aforementioned conditions. Cell culture supernatants and cell pellets were obtained 48 h after stimulation and stored at −80°C.

**Statistical analysis**

Statistical analysis was performed using statistical analysis software package (SPSS version 15.0, SPSS Inc, Chicago, IL, USA). Data were tested for normal distribution, homoscedasticity and random distribution. Comparisons between groups for baseline parameters were made with repeated measures analysis of variance (ANOVA; for parametric and log-transformed parametric data) or Kruskal-Wallis test (for data with no normal distribution). Trends over time were calculated with paired tests (paired Student’s t-test for parametric data and Wilcoxon test for non-parametric data). The significance of associations among various measures was determined by determination of intracellular ferritin concentrations which were significantly higher than in the control patients (P = 0.01, Figure 1C).

We then studied whether differences in the concentration of the master regulator of iron homeostasis, hepcidin, may affect the iron loading of monocytes. At baseline, patients had mean hepcidin serum concentrations of 15.15 ± 10.40 pmol/mL (mean ± SD). When we compared intracellular ferritin levels after iron administration according to baseline hepcidin levels, we found that patients with serum hepcidin levels >12 pmol/mL presented with significantly higher intracellular ferritin concentrations 48 h after iron administration (P = 0.02) than patients with low serum hepcidin baseline levels (<12 pmol/mL), whereas no difference was evident before iron administration (P = 0.72) (Figure 1D). In addition, we found a strong correlation between serum hepcidin and serum ferritin baseline levels (r = 0.737, P < 0.0001), while no significant association was found between serum hepcidin and markers of inflammation, such as C-reactive protein (r = 0.008, P = 0.97), serum TNF-α (r = 0.166, P = 0.485) or serum IL-6 (r = 0.164, P = 0.49) concentrations, respectively. In addition, neither serum IL-6 (r = 0.333, P = 0.07) nor serum TNF-α (r = 0.355, P = 0.13) correlated with serum ferritin levels at baseline.

This indicates that in this group of dialysis patients, serum hepcidin and ferritin levels rather reflected circulating iron levels than an underlying subtle immune activation.

**Results**

**Evaluation of baseline parameters**

Baseline and laboratory parameters of subjects from both groups are shown in Table 1. Forty-eight hours after the administration of a single dose of 100-mg iron sucrose, we found a significant increase in serum ferritin (P < 0.001) levels in the intervention group but not in controls. To study the effects of iron administration on iron metabolism of circulating monocytes, we quantified the expression of critical iron metabolism genes in isolated monocytes by means of RT–PCR. At baseline, we observed no differences in the mRNA expression of the iron uptake protein, transferrin receptor-1 (TfR-1) and for the only known iron exporter ferroportin-1 (Fpn-1) between the two groups (Figure 1). Forty-eight hours after iron administration, higher TfR-1 mRNA levels (P = 0.01) were found in monocytes from controls than in iron-treated patients, while Fpn-1 mRNA levels increased with iron treatment but slightly decreased in controls. The decrease in TfR-1 mRNA and increase of Fpn-1 mRNA levels in monocytes of iron-treated patients were indicative for iron accumulation, which was confirmed by determination of intracellular ferritin concentrations which were significantly higher than in the control patients (P = 0.01, Figure 1C).

Intravenous iron administration modulates monocyte immune effector function

Based on the known interaction of iron with immune function [9], we next studied the effects of iron treatment on monocyte effector pathways. Primary monocytes were isolated before iron injection at Day 0, and 48 h and 1 week after the administration of iron sucrose, respectively, and kept in tissue culture for 48 h. While no differences in concentrations of TNF-α, IL-6 and IL-10 in monocyte supernatants became evident at baseline, we observed higher concentrations of TNF-α and a significant rise in IL-6 levels in monocyte supernatants collected 48 h after iron treatment as compared with controls (Figure 2). This increase in TNF-α and IL-6 expression was transient, as levels returned to baseline 1 week after iron administration (data not shown). Considering these results, we wondered whether the modulation of monocyte immune effector pathways due to iron treatment differs as a function of baseline serum ferritin levels.

Role of pre-treatment ferritin levels on iron-mediated immune regulation

We thus compared patients from the intervention group according to their baseline serum ferritin levels. Patients
were dichotomized into a low (serum ferritin <170 μg/L) and a high ferritin (serum ferritin >350 μg/L) group (Table 2). While 48 h after iron injection only serum ferritin levels were significantly different between the low and high ferritin group (P = 0.001), serum iron levels and transferrin saturation increased significantly from Day 0 to 48 h (P = 0.02 and P = 0.03) only in patients from the low baseline ferritin group (Table 2).

When studying cytokine formation, we detected higher baseline concentrations of TNF-α, IL-6 and IL-10 in supernatants of isolated monocytes obtained from patients with low serum ferritin. In addition, TNF-α formation increased from baseline to 48 h after intravenous iron treatment in monocytes from the low ferritin group (P = 0.04) (Figure 3). Forty-eight hours after iron treatment, we observed higher IL-10 formation by monocytes obtained from patients of the low ferritin group as compared with monocytes from the high ferritin group (P = 0.01).

Intravenous iron administration leads to a transient increase in pro-inflammatory cytokine expression of circulating blood monocytes via the activation of NFκB pathway

We then studied potential mechanisms underlying increased formation of TNF-α by blood monocytes after iron treatment. We found the NFκB pathway and specifically phospho-NFκB-p65 levels to be activated in monocytes following iron treatment. Interestingly, the activation of NFκB pathway was more pronounced in patients having low serum ferritin levels at baseline. In contrast, analyses of the NFκB pathway in monocytes isolated from patients not receiving iron demonstrated
Fig. 2. Intravenous iron treatment promotes monocyte cytokine formation ex vivo. Monocytes were isolated from peripheral blood of patients for investigation of immune response ex vivo at baseline and 48 h after injection of iron (intervention) or saline (control). The concentrations of (A) TNF-α, (B) IL-6 and (C) IL-10 were determined in unstimulated monocytes after 48 h of culture. Boxes show means ± SEM; P-values are shown as determined by t-test.
**Table 2. Impact of iron treatment on haematological parameter according to baseline serum ferritin levels (means ± SD)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>48 h</td>
<td>Low vs. high serum ferritin</td>
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<tr>
<td>Low serum ferritin (&lt;170 μg/L) (n = 5)</td>
<td>187 ± 38</td>
<td>311 ± 64</td>
<td>0.76</td>
</tr>
<tr>
<td>High serum ferritin (&gt;350 μg/L) (n = 10)</td>
<td>211 ± 64</td>
<td>310 ± 67</td>
<td>0.43</td>
</tr>
<tr>
<td>Mean rhErythropoietin dosage before study entry (IE/week)</td>
<td>3600 ± 4930</td>
<td>3100 ± 9460</td>
<td>0.09</td>
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</tbody>
</table>

Data are shown as means ± SD. Significance reports differences according to baseline serum ferritin levels as well as changes in the low and high ferritin group from Day 0 (prior iron treatment) to 48 h after iron treatment. P-values are shown as determined by ANOVA, Kruskal–Wallis test or chi-square test for comparisons at baseline and 48 h after intervention. P-values for time courses were calculated with paired t-test or Wilcoxon test; P < 0.05 was considered to be significant. Conversion factors for units: serum iron in μg/dL to μmol/L, ×0.179.

**Discussion**

Herein, we investigated the effects of intravenous iron towards modulation of monocyte homeostasis and immune effector function. We could demonstrate that both pre-therapy ferritin levels, as an indicator of iron loading, and therapeutic application of iron exert subtle effects on monocyte immune responses.

Ferritin determination in serum is regarded as an important clinical parameter to assess body iron stores in haemodialysis patients [1,26]. Although serum ferritin levels correlate to body iron stores, one has to consider that ferritin

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a slight reduction in phospho-NF-κB-p65 levels from Day 0 to 48 h (Figure 4).

**Increased circulating ferritin concentrations are associated with an impaired immune effector function of blood monocytes**

As the impact of intravenous iron on monocyte immune function was related to differences in patients’ baseline serum ferritin levels, we speculated whether chronic iron overload as reflected by elevated serum ferritin levels might interfere with the immune effector function of circulating monocytes as well. Therefore, we investigated peripheral monocytes ex vivo following stimulation with interferon-gamma (IFN-γ) and LPS (Figure 5).

When studying the expression of Tlr-1 and Fpn-1 mRNA in monocytes from patients with low and high baseline ferritin levels, no significant differences were observed. In contrast, when measuring cytokine levels in culture supernatants, we observed significantly higher TNF-α, IL-6 and IL-10 concentrations when serum baseline ferritin levels were low (Figure 5). Accordingly, stimulation of the latter monocytes with IFN-γ or LPS resulted in significantly higher levels of IL-6 (P = 0.04) and IL-10 (P = 0.004) as found in monocytes from the high ferritin group, while the difference in TNF-α formation (P = 0.01) was only significant upon addition of LPS (Figure 5C–E).
Fig. 3. Effect of intravenous iron administration on monocyte iron homeostasis and cytokine formation depending on baseline serum ferritin levels. Patients of the intervention group ($n = 15$) were divided into low (serum ferritin <170 ng/mL; $n = 5$, black bars) and high ferritin group (serum ferritin >350 ng/mL; $n = 10$, white bars) according to the serum ferritin levels at study entry, and cytokine formation in supernatants of isolated monocytes was determined as detailed in the legend in Figure 2. P-values are shown as determined by paired t-test.
expression is induced by several inflammatory cytokines [17,27,28]; thus, serum ferritin levels during inflammation, as observed in many dialysis patients, do not exactly reflect body iron stores [29]. Therefore, serum ferritin-guided iron supplementation strategies harbour some uncertainties [17,27] and may impact on immune function, intravascular radical formation, and the risk for infections [19,20,30,31].

Monocytes and macrophages play key roles in the regulation of iron homeostasis since they re-deliver iron to the circulation which was regained from senescent erythrocytes following erythrophagocytosis. Under chronic inflammatory conditions—as they are frequently present in dialysis patients—the uptake of iron into monocytes is significantly increased, and the transfer of the metal to the circulation is reduced as a consequence of cytokine-mediated regulation of iron transport [23]. A part of this effect can also be referred to the function of the master regulator of iron homeostasis, hepcidin, which is induced by iron overload and inflammation [24,32,33]. Hepcidin exerts its biological function by interacting with the iron export protein Fpn-1 leading to Fpn-1 internalization, degradation and blockage of iron export [32,33]. Importantly, increased circulating concentrations of hepcidin, which are a reflection of chronic iron overload, inflammation and decreased urinary excretion of the peptide, have been observed in dialysis patients [16,20,34–36]. Hepcidin interacts with Fpn-1 on monocytes/macrophages, thus inhibiting the transfer of iron which has been acquired by these cells following therapeutic iron supplementation [32,33,37]. This will lead to limitation of iron availability for erythropoiesis, which will be more pronounced with a more advanced immune activation [9]. This notion goes along with the observation that a more advanced inflammation is associated with severe anaemia, a poor response to iron supplementation strategies and an impaired response to recombinant erythropoietin [9,17,27,38–41]. Interestingly, we found that pre-treatment serum hepcidin levels were positively associated with iron retention in monocytes following therapeutic application of the metal. This indicates that circulating hepcidin concentrations may determine the availability of iron for erythropoiesis by modulating iron uptake and retention in monocytes. This is not only of interest in terms of pathophysiology, but also determination of hepcidin in renal disease patients may turn out as a clinically valuable marker to predict the erythropoietic response to iron administration [24,32–37].

Contrariwise, uptake and retention of iron by monocytes/macrophages will impact on immune function and host responses towards invading pathogens. When studying spontaneous and inflammation-inducible cytokine formation by isolated monocytes, we found that pro-inflammatory immune pathways such as formation of IL-6 or TNF-α were significantly reduced in subjects with high (>350 ng/ml) as compared with subjects with lower (<170 ng/ml) serum ferritin levels. As we excluded an underlying state of inflammation by means of clinical and laboratory testing, this is most likely a reflection of iron-mediated regulation of cellular immunity, which has also been observed in dialysis patients [20]. Thereby, iron inhibits IFN-γ-mediated immune effector pathways in monocytes leading to diminished formation of TNF-α, nitric oxide or oxygen radicals [42,43]. In addition, iron overload significantly impairs the functionality of neutrophil granulocytes in dialysis patients [44]. Thus, iron status may determine the susceptibility to infection in dialysis patients [19,45].

Accordingly, prospective studies of bacterial infections in chronic haemodialysis patients demonstrated an increased incidence of bacteraemia in patients receiving iron who had high serum ferritin levels at baseline [45], though ferritin baseline levels were not found to be different in end-stage renal disease patients with or without bacteraemic episodes [46]. However, a previous study demonstrated a significant impairment of neutrophil function in dialysis patients with ferritin levels >650 ng/mL [44]. Moreover, a
Fig. 5. Impact of serum ferritin on the expression of critical iron genes and immune response in monocytes of dialysis patients. Patients were divided into low (serum ferritin < 170 ng/mL; n = 5, black bars) and high ferritin group (serum ferritin > 350 ng/mL; n = 10, white bars) according to baseline ferritin levels. Monocytes were isolated and left untreated (control), or stimulated with 500 U/mL rh-interferon-gamma (IFN-γ) or 500 ng/mL LPS for 48 h. (A, B) RT-PCR quantification of (A) transferrin receptor-1 (TfR-1) cDNA and (B) ferroportin-1 (Fpn-1) cDNA expression in monocytes, and ELISA quantification of (C) TNF-α, (D) IL-6 and (E) IL-10 levels in cell culture supernatants was then performed. For graphical purposes, data from LPS-stimulated monocytes are shown with a different ordinate (ordinate at right side of the figure) than data from unstimulated and IFN-γ-stimulated cells (ordinate at left side). Boxes show means ± SEM. P-values are shown as determined by t-test.
retrospective analysis of dialysis patients indicated that those with repleted iron stores before the initiation of iron therapy had a 2.5-fold higher risk ratio for bacteremia during a follow-up period of 1 year [47]. This may not only be due to the effects of iron on immune function but also refer to the fact that iron is essential for the proliferation and pathogenicity of microbes [19,48]. Thus, although being safe in terms of immediate toxicity and side effects, iron therapy in dialysis patients harbours potential hazards which are not fully understood and recognized so far [31,49].

Interestingly, we also found that a single dose of iron sucrose causes a transient increase in the formation of IL-6 and TNF-α by isolated monocytes. This could be traced back to activation of the NFκB pathway in circulating blood monocytes, which is most likely a consequence of iron acquisition and subsequent expansion of the cytoplasmic labile iron pool [50], because activation of the NFκB pathway by iron has been described previously [51,52]. Notably, we found that this effect was transient, and its termination may be explained by incorporation of iron into ferritin and/or release of the metal from cells [50].

In summary, we have demonstrated that therapeutically administered iron induces an increase in intramonicoytic ferritin levels, thus may be taken up and stored by monocytes. While iron exerts short-term pro-inflammatory effects via the activation of the NFκB pathway, cumulative iron loading negatively impacts on innate immune effector function of patients and likewise increases the risk for infectious complications. Hence, accurate monitoring of body iron status in dialysis patients is mandatory to warrant an appropriate treatment of renal anaemia, an issue which is only insufficiently fulfilled currently by the determination of the iron and inflammation-inducible protein ferritin. Thus, we urgently need new markers of iron homeostasis which clearly indicate the need of iron for erythropoiesis in order to guarantee optimal treatment of renal anaemia while avoiding detrimental effects of iron overload [9,24,29,36].

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Iron therapy and immune function


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