

Impairment of Transendothelial Leukocyte Migration by Iron Complexes

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Abstract. Although iron sucrose and iron gluconate are generally well tolerated in patients who are treated for renal anemia, recent clinical studies and cell culture experiments suggested significant toxicity and long-term side effects arising from the use of these iron complexes. Because of the possible role of iron in infection or cardiovascular disease, it was theorized that parenteral iron compounds influence endothelial and PMN interaction *in vitro*. A well-established double-chamber method was used to assess the effect of different concentrations of iron sucrose and iron gluconate (1, 25, 50, and 100 $\mu\text{g/ml}$) on the transendothelial migration of PMN. Preincubation of PMN and endothelial cells as well as preincubation of PMN alone with 25, 50, or 100 $\mu\text{g/ml}$ iron resulted in a significant decrease in PMN migration. In contrast,

after incubation of the endothelial cells alone with iron, no reduction in the transendothelial migration of PMN was observed. Preincubation of PMN and/or endothelial cells with 1 $\mu\text{g/ml}$ iron did not lead to any decrease in the rate of migrated PMN. The only significant change in experiments with 1 $\mu\text{g/ml}$ was an increase in PMN migration after preincubation of endothelial cells and PMN with iron gluconate. A four-way ANOVA showed a significant effect of the iron concentration ($P < 0.000001$), of type of iron complex ($P < 0.005$), of the preincubation of endothelial cell ($P < 0.001$), and of the preincubation of PMN with iron ($P < 0.000001$) on PMN diapedesis. It is concluded that iron sucrose and iron gluconate cause a significant inhibition of transendothelial migration of PMN.

Renal anemia therapy requires an intravenous iron substitution in addition to the erythropoietin therapy in the majority of patients (1). Iron substitution not only reduces the erythropoietin dosage needed but also is necessary to maintain the target hemoglobin above 11 g/dl (2,3). There are several iron preparations for intravenous use available, all of which have potential side effects, such as allergic reactions, cell injury, or endothelial dysfunction (4–7). Moreover, iron therapy may be associated with infectious complications and with loss of the ability of patient serum to resist the bacterial growth (8–10). PMN play a vital role in the nonspecific immune reaction against bacterial infections executing functions such as chemotaxis, transendothelial migration, phagocytosis, and intracellular killing by proteolytic enzymes or toxic oxygen radicals. Although the effects of iron on chemotaxis of PMN, phagocytosis, and intracellular killing in PMN were studied previously, the effect of iron complexes on PMN–endothelial cell interaction is unknown. Therefore, we examined the effect of incubation of PMN and/or endothelial cells with two widely used iron complexes, iron(III)-hydroxide-sucrose complex (iron sucrose) and iron(III)-sodium-gluconate in sucrose (iron glu-

conate), on the PMN migration through the endothelium in an *in vitro* setting.

Materials and Methods

Reagents

Fibronectin and endothelial cell growth supplement were purchased from Collaborative Biomedical Products (Bedford, MA). FCS, collagenase type I, Hank's solution, Dulbecco's PBS, and RPMI 1640 medium were obtained from Life Technologies Laboratories (Grand Island, NY). Ficoll-Paque was obtained from Pharmacia (Uppsala, Sweden). Calcein AM was from Molecular Probes (Eugene, OR), iron(III)-sodium-gluconate in sucrose (iron gluconate, Ferrlecit) was from Rhone-Poulenc (UK), and iron(III)-hydroxide-sucrose complex (iron sucrose, Ferrivenin) was from Laevosan (Austria).

Endothelial Cell Culture

Human umbilical cord vein endothelial cells (HUVEC) were cultured according to Jaffe *et al.* (11) with slight modifications. Briefly, cells were pooled after collagenase treatment and seeded on six-well cell culture plates coated with fibronectin (2.5 $\mu\text{g/cm}^2$). Cells were grown in RPMI 1640 medium supplemented with 20% FCS, 1% L-glutamine, 100 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin, and 1 $\mu\text{g/ml}$ Fungizone and after the first day with 5% FCS under standard cell culture conditions (humidified atmosphere, 5% CO_2 , 37°C). Reaching confluence within 3 to 4 d, primary endothelial cell cultures were subcultured and cells from second through fifth passages were used for the migration experiments. Endothelial cells in each passage showed cobblestone morphology and acetylated LDL uptake and expressed von Willebrand factor, CD31, and angiotensin-converting enzyme (data not shown).

Transendothelial PMN Migration

Isolation of PMN was performed as described previously (12). In brief, low-molecular weight heparin treated blood obtained from

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healthy volunteers was layered over Ficoll-Paque (1.077 g/ml). After an initial incubation step of 45 min at room temperature, supernatants were layered on 63% Percoll underlain with 72% Percoll. After centrifugation at $500 \times g$ for 25 min at room temperature, cells were washed twice in Ca^{2+} and Mg^{2+} free Hank's solution. The cell pellets were resuspended in RPMI 1640 medium at a final concentration of $2.5 \times 10^6/\text{ml}$. HUVEC (passages 2 to 5) were transferred and grown on fibronectin-coated permeable membrane inserts (diameter, 9 mm; pore size, $3.0 \mu\text{m}$; Falcon/Becton Dickinson, Mountain View, CA) of a 24-multiwell double-chamber system.

Confluent endothelial cell monolayers and/or PMN (5×10^5 cells) were incubated first with iron sucrose or iron gluconate in four different concentrations (1, 25, 50, and $100 \mu\text{g}/\text{ml}$ corresponding to 1.79, 44.75, 89.5, and $179 \mu\text{mol}/\text{L}$, respectively; $\mu\text{mol}/\text{L} = 0.179 \times \mu\text{g}/\text{dl}$) or medium (control) for 1 h at 37°C . PMN were added into the upper chamber and were allowed to migrate through the endothelial cell monolayer into the lower chamber for 2 h at 37°C . We used formyl-methionyl-leucyl-phenylalanine as a chemoattractant in a concentration of 10^{-8} M in the lower chamber. The iron complexes were dissolved in RPMI 1640 medium to make solutions of 10, 250, and $500 \mu\text{g}/\text{ml}$ and 1 mg/ml. The final concentrations were reached after a further 1:10 dilution. All iron solutions in their final concentrations had pH values ranging from 7.2 to 7.9.

At the end of incubation, the membrane inserts that contained the endothelial cell monolayers as well as the nonmigrated leukocytes were discarded. Without further washing or transfer steps, migrated PMN in the lower chamber were immediately exposed to the fluorescent dye calcein AM (2 mM). After incubation in the dark at room temperature for 30 min under mild shaking, the relative fluorescence intensity was measured using the cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA). Absolute cell counts were determined by comparison with dilution series of calcein AM-labeled PMN cultured in RPMI 1640 medium as described (13). Each experiment was performed 6 to 10 times in triplicate. Cell viability for the endothelial cells and PMN in all experiments was $>95\%$ as determined by trypan blue exclusion.

Experimental Design

For studying the transendothelial migration of PMN, we used a well-established double-chamber system (13,14). After preincubation of endothelial cells and/or PMN with iron sucrose or iron gluconate at four different concentrations, PMN were allowed to migrate through the endothelial cell layer and counted. For each set of experiments, we used PMN obtained from a single healthy donor (age 25 to 35, male or female) and endothelial cells from the same preparation to allow for a valid comparison of both iron complex types (endothelial cells with iron treatment, PMN with iron treatment, PMN and endothelial cells with iron treatment). We carried out 6 to 10 experiments for each of the four iron complex concentrations used (control and 1, 25, 50, and $100 \mu\text{g}/\text{ml}$), all in triplicate. Finally, we analyzed 184 mean values of triplicates.

Statistical Analyses

The number of migrated PMN in the control experiments served as baseline and was taken as reference (100%). The number of migrated PMN in all other settings is expressed as percentage of the baseline and is given as mean \pm SD. The *t* test was used as appropriate for group comparisons.

We performed a four-way ANOVA to examine independent effects of iron sucrose or iron gluconate, of the dose of iron (1, 25, 50, and $100 \mu\text{g}/\text{ml}$), of the preincubation of the endothelial cells with iron

(yes/no term), and of the preincubation of PMN with iron (yes/no term) on the PMN function. We also assessed interactions of the aforementioned factors.

In addition, we performed separate analyses for each iron brand (iron sucrose or iron gluconate) by three-way ANOVA with the variables iron concentration (1, 25, 50, and $100 \mu\text{g}/\text{ml}$), the preincubation of the endothelial cells with iron (yes/no term), and the preincubation of PMN with iron (yes/no term) including interactions among these factors. Statistical analysis was performed by Statistica for Windows 5.1 (Stat Soft, Inc., Tulsa, OK). The analysis of pooled data was performed by grouping all data from experiments with both iron brands and by grouping data from each iron brand separately.

Results

Univariate Analyses

The simultaneous preincubation of PMN and endothelial cells as well as preincubation of PMN alone with 25, 50, or $100 \mu\text{g}/\text{ml}$ iron resulted in a significant decrease in PMN migration (Table 1). In contrast, after incubation of the endothelial cells alone with iron, we observed no reduction in the transendothelial migration of PMN. Preincubation of PMN and/or endothelial cells with $1 \mu\text{g}/\text{ml}$ iron did not lead to any decrease in the rate of migrated PMN. The only significant change in experiments with $1 \mu\text{g}/\text{ml}$ was an increase in PMN migration after preincubation of endothelial cells as well as PMN with iron gluconate (Table 1).

Multivariate Analyses

A four-way ANOVA of all 184 mean values of triplicates showed a significant effect of the iron concentration ($P < 0.000001$), of the iron brand (greater decrease of PMN migration with iron sucrose, $P < 0.005$), of the preincubation of endothelial cells with iron (increase of PMN migration with iron, $P < 0.001$), and of the preincubation of PMN with iron (decrease of PMN migration with iron, $P < 0.000001$) on the transendothelial migration of PMN. We observed a significant interaction of iron brand \times preincubation of endothelial cells with iron ($P < 0.05$), of iron concentration \times preincubation of PMN with iron ($P < 0.00001$), and of preincubation of endothelial cells with iron \times preincubation of PMN with iron ($P < 0.05$).

We also conducted separate analysis for each iron complex. The analysis of iron sucrose by three-way ANOVA demonstrated that the iron concentration ($P < 0.000001$) and the preincubation of PMN with iron ($P < 0.000001$) had a major inhibitory effect on the transendothelial migration of PMN. In addition, we observed a significant interaction of iron concentration with preincubation of PMN with iron ($P < 0.0001$) and preincubation of endothelial cells with preincubation of PMN ($P < 0.05$). The analysis of iron gluconate revealed that the iron concentration ($P < 0.0005$), preincubation of PMN with iron ($P < 0.05$), and the interaction of iron concentration with preincubation of PMN ($P < 0.05$) significantly inhibited the PMN migration.

Analysis of Pooled Data

When both PMN and endothelial cells were incubated with iron concentrations $>1 \mu\text{g}/\text{ml}$, we observed a significantly

Table 1. Transendothelial PMN migration after preincubation of endothelium and/or PMN with iron sucrose or iron gluconate (percentage of control [EC-/PMN-], mean \pm SD)^a

Cell Types Preincubated with Iron Complexes	Iron Concentration (μ g/ml)	Migration of PMN with Iron Sucrose (% of Control)	Iron Sucrose versus Control (P)	Migration of PMN with Iron Gluconate (% of Control)	Iron Gluconate versus Control (P)	Iron Gluconate versus Iron Sucrose (P)
EC+/PMN+	1	126 \pm 42.6	NS	142.1 \pm 34.4	<0.05	NS
	25	33.5 \pm 12.2	<0.002	58.8 \pm 18.7	<0.05	<0.05
	50	27.9 \pm 18.6	<0.0005	81.8 \pm 33.0	NS	<0.005
	100	31.9 \pm 24.5	<0.0005	88.4 \pm 40.4	NS	<0.02
EC-/PMN+	1	138.8 \pm 38.5	NS	123.6 \pm 24.1	NS	NS
	25	45.8 \pm 20.6	<0.02	56.0 \pm 32.9	NS	NS
	50	38.2 \pm 24.9	<0.002	57.1 \pm 18.1	<0.005	<0.05
	100	37.1 \pm 27.4	<0.001	48.6 \pm 28.7	<0.005	NS
EC+/PMN-	1	152.5 \pm 63.3	NS	164.1 \pm 100.1	NS	NS
	25	127.7 \pm 34.4	NS	116.9 \pm 28.1	NS	NS
	50	113.7 \pm 53.7	NS	146.2 \pm 49.9	NS	NS
	100	92.6 \pm 40.3	NS	128.8 \pm 37.4	NS	NS

^a EC+, endothelial cells with iron preincubation; EC-, endothelial cells without iron preincubation; PMN+, PMN with iron preincubation; PMN-, PMN without iron preincubation.

impaired migration of PMN (pooled data from experiments with both iron brands at 25, 50, or 100 μ g/ml as percentage of control: 46.2 \pm 19.9%, $P < 0.0005$; 54.5 \pm 37.7%, $P < 0.0015$; 60.1 \pm 43.5%, $P < 0.005$, respectively). Exposure of PMN to iron concentrations >1 μ g/ml resulted in a significant decrease in PMN migration (pooled data from experiments with both iron brands at 25, 50, or 100 μ g/ml as percentage of control: 50.9 \pm 26.9%, $P < 0.001$; 47.7 \pm 23.0%, $P < 0.00001$; 42.8 \pm 27.6%, $P < 0.000005$, respectively). There was no significant difference in PMN migration when only endothelial cells were preincubated with iron.

The analysis of pooled experiments with iron sucrose or iron gluconate separately in concentrations ≥ 25 μ g/ml ($n = 24$ in each group) showed that the preincubation of endothelial cells and PMN resulted in a profound decrease of PMN migration (to 30.9 \pm 19.2% with iron sucrose and to 78.8 \pm 34.1% with iron gluconate from control experiments without iron). The difference between iron sucrose and iron gluconate groups was significant ($P < 0.00002$). Preincubation of PMN with iron resulted in a significant decrease of PMN migration in the iron sucrose group (39.5 \pm 23.8%, compared with control, $P < 0.000001$) and the iron gluconate group (53.3 \pm 25.1%, compared with control, $P < 0.000002$), which was also different between the two groups ($P < 0.002$). Although preincubation of endothelial cells alone with iron sucrose showed no change of PMN migration (108.3 \pm 44.1%, compared with control), iron gluconate showed a slight stimulatory effect (132.1 \pm 39.8%, compared with control, $P < 0.005$). This effect, however, was statistically NS between the two groups.

Discussion

We provide evidence that two widely used iron complexes, iron sucrose and iron gluconate, compromise transendothelial migration of PMN. Iron treatment of PMN alone or the simul-

taneous exposure of endothelial cells and PMN to iron led to a significant decrease of PMN migration.

The safety and efficacy of iron sucrose and of iron gluconate for treatment or prevention of iron deficiencies among renal failure patients was recently summarized by Yee and Besarab (15) and by Fishbane and Wagner (16). Several authors reported a variable incidence of side effects of iron sucrose (15,17–20) and of iron gluconate (16,21–23) in hemodialysis patients. In patients who were treated with iron sucrose, the incidence of severe adverse events resulting in stopping of further treatment ranged from 0 to 0.26% at iron doses of 10 to 300 mg per infusion (17–20,24). Severe adverse events occurred in 3 to 36% of patients who received 400 and 500 mg per infusion (18). The proportion of patients who experienced adverse events that did not preclude further therapy ranged from 0 to 8.7% at doses up to 100 mg per infusion (17–20,24). In contrast, therapy with iron gluconate was associated with severe adverse events in up to 3% of patients who were exposed to 62.5 to 250 mg of iron per infusion, and adverse events were observed in only 3.9% across the same dose range (21–23,25). It is interesting that high doses of 312.5 to 500 mg of iron gluconate were not associated with any severe adverse events in a small series of hemodialysis patients (25).

Furthermore, *in vitro* studies showed that iron sucrose and iron gluconate conferred cytotoxic effects on mouse proximal tubule cells, on HK-2 cells, and on bovine endothelial cells as measured by LDH release and by tetrazolium dye assay (MTT uptake) (7). Other studies demonstrated that nutritional iron overload increased the abundance of the reactive oxygen species in rats (26). These reactive oxygen species led to a sequestration of nitric oxide and to a compensatory upregulation of renal endothelial nitric oxide synthase and inducible nitric oxide synthase expression. Furthermore, hydroperoxide-in-

duced oxidative stress and endothelial cell apoptosis require iron uptake via the transferrin receptor pathway (27).

Clinically, iron sucrose infusion was shown to increase the level of bleomycin-detectable iron in patients without ESRD (28). A comparable increase of non–transferrin-bound iron was also reported for hemodialysis patients who received 100 mg of iron sucrose (29). An increase of non–transferrin-bound iron was also associated with a substantial decrease in flow-mediated vasodilation and an increase of superoxide generation in whole blood of healthy volunteers after the infusion of 100 mg of iron sucrose (5). However, it is noteworthy that these studies did not make side-by-side comparisons of iron sucrose with iron gluconate. The studies by Kooistra *et al.* (29) and Roob *et al.* (30) showed an increase of serum iron ranging from 75 to 240 $\mu\text{mol/L}$, which is comparable to the iron concentrations used in our study.

Gaenger *et al.* (31) examined vascular function in 41 iron-overloaded patients who had hereditary hemochromatosis and in 51 matched control subjects. Endothelial-dependent vasodilation was impaired and intima media thickness of the carotid arteries was increased in these patients. Treatment with repeated phlebotomy resulted in an improvement of vascular function in male patients with hereditary hemochromatosis. Similarly, Duffy (32) showed that iron chelation with deferoxamine improved nitric oxide-mediated, endothelium-dependent vasodilation in patients with coronary artery disease. Several studies suggested that iron enhances oxidative stress and increases the risk for cardiovascular disease (33,34). This assumption is indirectly supported by the studies of Drüeke *et al.* (35) and Patruta *et al.* (36). The first study showed that advanced oxidation protein products correlate with iron exposure and with carotid artery intima thickness of dialysis patients, whereas the latter demonstrated an enhanced oxidative burst in PMN of hemodialysis patients with ferritin levels above 650 $\mu\text{g/L}$. These data support the concept that iron complexes may also exert their negative effects on endothelial function by enhancing oxidative stress to plasma lipids or proteins. In our study, there was no major direct effect on transendothelial PMN migration after exposure of endothelial cells alone to iron complexes. The only effect of iron on endothelial cells was a slight increase of PMN migration. However, there are conflicting data on the correlation between iron therapy and risk for cardiovascular disease. The National Health and Nutritional Examination Survey study demonstrated that greater iron intake was associated with reduced coronary artery disease (37). Another study showed that as serum iron levels increased, risk for mortality from cardiovascular disease decreased (38). In a study investigating autopsy data from patients with hemochromatosis, only 12% of patients with iron overload had advanced to severe coronary artery disease as compared with 33% of the matching control subjects without any signs of iron overload (39).

The effect of iron therapy on the risk for infection is another controversial issue. The review by Patruta and Hörl (8) stated that iron overload is a risk factor for infection. *In vitro* evidence for impaired PMN function as a consequence of chronic iron therapy was obtained by the same group (36). This study

showed that PMN from hemodialysis patients who were treated with low-dose iron sucrose (10 mg three times weekly after hemodialysis) and a ferritin level $>650 \mu\text{g/L}$ presented with impaired intracellular killing as well as phagocytosis, as compared with PMN harvested from healthy subjects. Furthermore, it was demonstrated that iron dextran attenuates PMN function in hemodialysis patients even without signs of iron overload and with normal iron indices at clinically relevant concentrations (40). No such studies are currently available for iron gluconate. In contrast to the above findings, several studies concluded that there was no increased infection rate resulting from iron therapy in patients without renal failure or in patients undergoing dialysis (41–43). Furthermore, in a study that compared effectiveness and safety of iron sucrose and iron gluconate, no difference was observed between these iron complexes (44).

Our study focuses on the impact of iron sucrose and iron gluconate on transendothelial PMN migration. The only evidence of an iron effect on PMN migration comes from a previous study with iron-overloaded homozygous β -thalassaemia patients. In this study, neutrophil migration impairment was shown in almost all of the patients who have experienced pyogenic infections (45). However, this study did not investigate the interaction of PMN with endothelial cells. Our study shows that exposure of PMN to iron resulted in a substantial impairment (down to 30% of control) of transendothelial migration at clinically relevant iron concentrations $>1 \mu\text{g/ml}$. These effects in our experiments were not related to reduced cell viability or pH fluctuations in cell culture medium after the addition of iron.

The main limitation in this study is that the effect of iron complexes is demonstrated in an *in vitro* setting, which may not reflect the exact circumstances *in vivo*. It is of interest that there is no information available on whether the iron complexes used in our study cleave into their iron and carbohydrate components or stay as a whole drug.

Our data suggest that parenterally used iron complexes impair transendothelial migration of PMN. This adds a new aspect to the well known effects of iron in modifying PMN or endothelial cell functions. The clinical implications of our finding, including the differences between iron sucrose and iron gluconate, are currently unknown and require further studies.

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