Non–Transferrin-Bound Iron in the Serum of Hemodialysis Patients Who Receive Ferric Saccharate: No Correlation to Peroxide Generation

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Abstract. Intravenous iron (iv.Fe) is used to optimize response to recombinant human erythropoietin (r-HuEPO) in ESRD, but no consensus exists with respect to the best regimen to avoid transferrin “oversaturation,” oxidative stress, and the occurrence of non–transferrin-bound iron (NTBI). Iv.Fe was stopped for 1 wk in 35 hemodialysis (HD) patients who were routinely receiving iv.Fe and r-HuEPO. The iv.Fe group received 100 mg of ferric saccharate (Venofer) at the end of the first HD session, whereas the time-control group was treated under the same conditions but received no iv.Fe. Serum samples were taken before the first HD session, immediately and 60 min after iv.Fe administration, and before the next HD session. Sera were analyzed for NTBI and peroxides; transferrin saturation was analyzed by urea-PAGE and Western blot. In an in vitro model system with HepG2 cells, the effects of HD serum on the labile iron pool (LIP) were assayed using the fluorescence calcein assay. NTBI significantly increased after iv.Fe-administration and returned to baseline values before the next HD-session. There was a shift from apotransferrin to monoferric transferrin, but no “oversaturation” of transferrin after iv.Fe-treatment. Peroxides increased in both groups after HD. Hemodialysis decreased bioavailable iron for the LIP in HepG2-cells, whereas serum of iv.Fe-treated HD patients highly increased the LIP in these cells. A total of 100 mg of iv.Fe led to NTBI generation but not to an oversaturation of transferrin. Peroxide concentrations significantly increased during HD but were not correlated to iv.Fe administration and seemed to result from other sources of oxidative stress related to HD. NTBI can enter liver cells and increase the potentially harmful LIP.

ESRD typically results in anemia, primarily as a result of deficient renal production of erythropoietin (EPO). Most patients who undergo hemodialysis (HD) therefore are treated with recombinant human erythropoietin (r-HuEPO) (1,2). The most important confounding factor limiting the effectiveness of rHuEPO is absolute or functional iron deficiency. Iron is usually substituted by intravenous iron (iv.Fe), because oral supplementation is inefficient (3–6). Despite the acknowledged safety of iv.Fe preparations such as ferric saccharate (7–9), the occurrence of non–transferrin-bound iron (NTBI) in the plasma of patients has been reported, either directly (10–14) or indirectly, by detection of enhanced symptoms of oxidative stress (15–18), of neutrophil damage (19), of support of bacterial growth (11), or increased incidence of infectious diseases (20,21).

There is wide agreement that patients who undergo regular HD treatment experience increased oxidative stress (22). NTBI in the circulation could in principle participate in redox reactions that give rise to reactive oxygen species. NTBI therefore is implicated to exacerbate oxidative stress in ESRD patients who receive iv.Fe therapy (23,24).

The chemical nature of NTBI is not really known. Binding to citrate or albumin was suggested by some authors (25–27). Although the binding affinity of most of them, such as albumin, is relatively low compared with transferrin, they are present at high concentrations, which makes them effective competitors with transferrin (28,29).

The preparations themselves do not yield iron to plasma transferrin in vitro (30,31); thus, processing by reticuloendothelial cells or by the liver presumably precedes iron release into the labile plasma iron pool. Recently, we showed the ability of parenteral iron preparations to deliver iron to nonreticuloendothelial cells (32) and their effect on the labile iron pool (LIP) of the human hepatoma cells HepG2 (33). Because elevations of the LIP are implicated in the generation of oxidative cell injury (34), these findings may have important implications on the possible toxicity of parenteral iron preparations. This is particularly true for liver hepatocytes, because the liver is also the main sink for excess iron either from transferrin or from non-transferrin sources.

ESRD patients usually have low plasma transferrin concentrations (35). Nevertheless, the transferrin saturation (TFS) is

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below the normal range. Reports on apparent oversaturation of transferrin in iv. Fe therapy (36–38) seem to rest on analytical errors in the determination of plasma iron (30,31).

There are two possibilities to measure NTBI: Indirect measurement involves the chemical reactivity of NTBI, for instance, the bleomycin method (39–41), whereas direct measurement typically involves the use of scavenging molecules to mobilize loose, nonspecifically bound iron (42,43). The results of such tests largely depend on the design of the assay. The recently described NTBI assays based on quenching the fluorescence of metal-binding dyes (12,13) open the possibility to obtain reliable results in statistically significantly great numbers, which does not require HPLC (44).

This study was designed to relate the levels of NTBI to TFS and the generation of peroxides in ESRD patients who undergo iv. Fe therapy under controlled conditions. In an in vitro model system with HepG2 cells using ESRD sera, the effect of dialysis and in vivo iv. Fe administration on the LIP was assayed using the fluorescence calcein assay.

Materials and Methods

Study Design

All chemicals were purchased from Sigma (St. Louis, MO) or Merck if not indicated otherwise. During this study, all patients underwent dialysis with an Asahi hollow fiber dialyzer APS 650 with biocompatible polysulfone membrane (γ-sterilized) and were heparinized with a high molecular weight heparin (Heparin Immuno; Baxter) by continuous intravenous infusion.

Parenteral iron administration was stopped 1 wk before the start of the study (washout phase). Thirty-five HD patients who routinely receive iv. Fe in combination with rHuEPO in the maintenance phase were included in this study after having given their informed consent. Patients were divided into two groups: The iv. Fe-group (27 patients) received an infusion of 100 mg of ferric saccharate (Venofer; Vifor Int., St. Gallen) during 10 min at the end of the HD session. This dose corresponds to the highest recommended single dose for this product (by the manufacturer). The time-control group (eight patients) was treated under the same conditions but received no iv. Fe. Four blood samples were collected (Figure 1): (1) before the first HD session; (2) directly after iv. Fe infusion (t = 0 min); (3) after 60 min (t = 60 min); and (4) before the next HD session, which was approximately 2 d after the first session. From the time-control group, serum samples were collected at the same times. Whole blood samples were drawn in Vacutette with Z Serum Separator clot activator (Greiner bio-one; Graz, Austria) and after 10 min were centrifuged at 2000 × g for 10 min. Serum samples were frozen immediately after centrifugation and kept at −20°C until analysis. All sera were analyzed for NTBI; the relative amount of apo-, monoferric-, and diferric transferrin; and the presence of peroxides. Sera from five ESRD patients from the iv. Fe group were also assayed for bioavailable iron by the fluorescence calcein assay (see below).

Laboratory Assessments

Clinical routine methods were used for the determination of serum iron (by the Guanidin-FerroZine-method, INTEDRA), serum ferritin (by enzyme immunoassay, COBASCORE), and serum transferrin concentration (by immunoturbidimetry, INTEDRA) in serum samples taken before the first hemodialysis session and analyzed in the clinical laboratory at the hospital (Table 1).

Determination of Peroxides

To avoid a loss of total peroxide activity during prolonged storage, serum samples were stored for no more than 1 wk at −20°C after collection. For determination of peroxide concentration, the “Peroxide-activity” assay (POX ACT; Tatzberger KEG, Klosterneuburg, Austria) was used, which is based on the reaction of horseradish peroxidase (HRP) with peroxides, using tetramethylbenzidine as the chromogen substrate. Ten microliters of the serum was incubated with a mixture of HRP, tetramethylbenzidine, and phosphate buffer for 20 min, and absorbances were determined in a multiwell plate reader (Victor from Wallac) at 450 nm. Using a hydrogen peroxide (H₂O₂) standard curve, total peroxide concentrations were expressed as micromoles of H₂O₂ equivalents per liter of serum.

Measurement of NTBI

NTBI was measured according to the method published by Breuer et al. (12), which is based on the binding of NTBI from the sera to...
Table 1. Baseline iron indices and clinical characteristics of HD patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (n)</th>
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<tbody>
<tr>
<td>Serum iron (μg/dl)</td>
<td>66.6 ± 6.1 (33)</td>
</tr>
<tr>
<td>Transferrin (g/L)</td>
<td>1.62 ± 0.25 (33)</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>29.4 ± 2.7 (33)</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>508 ± 66 (33)</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>13.5 ± 3.5 (32)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>15 (35)</td>
</tr>
<tr>
<td>Smokers</td>
<td>8 (35)</td>
</tr>
</tbody>
</table>

* Data are expressed as mean ± SE.

a In two patients, no baseline iron indices were determined; C-reactive protein was not determined in three patients.

Fluorescence Calcein Assay for ESRD Serum Samples

The influence of ESRD serum on the LIP was assayed by the fluorescence calcein assay (49). Human hepatoma HepG2 cells were cultured in DMEM containing 10% FCS, 2 mmol/L L-glutamine, and 50 μg/ml gentamicin on 48-well tissue culture plates at a density of 1 × 106 cells/ml. After 2 d, the cells were in the log phase and were used for the measurement of the LIP.

Cells were incubated with ESRD sera for 2.5 h at 37°C. Then the cells were washed with DMEM containing 50 μmol/L DTPA and two more washings with DMEM alone to remove surface-bound iron. The cells were subsequently loaded with 0.25 μmol/L calcein-AM for 15 min at 37°C in DMEM buffered with 20 mmol/L Hepes. The cell monolayer was washed free of excess calcein-AM and reincubated with DMEM containing 20 mmol/L Hepes and a fluorescence-quenching anti-calcein antibody (10 μl/ml medium) to eliminate all extracellular fluorescence (33). Calcein fluorescence was measured in a fluorescence plate reader (VICTOR II; Perkin Elmer; excitation/emission filters of 485/530 nm). The DFO-coated 96-well plates were prepared in advance by incubation of the plates with 0.1 ml of 75 mg of desferrioxamine (DFO)-coated wells in the presence of a mobilizing agent (oxalate). The binding of NTBI to DFO in the wells is detected with a nonfluorescent iron-calcein complex, which can donate iron to DFO not occupied by NTBI from the serum sample. Because calcein has a lower affinity to iron than DFO, calcein is iron-free and the fluorescence of free calcein can be measured.

Statistical Analyses

Statistical analysis was performed with GraphPad Prism software. Data are presented as mean ± SEM unless stated otherwise. P < 0.05 was considered significant. A paired t test was applied for analyzing differences to the baseline during the treatment in one group. Significant differences to the baseline are marked in the figures with * (P < 0.05), ** (P < 0.01), and *** (P < 0.001). An unpaired t test was used for analyzing differences between iv.Fe-treated and the time-control group. Significant differences are marked with + (P < 0.05), ++ (P < 0.01), and +++ (P < 0.001) in the figures.

Results

Effect of Dialysis and Iv.Fe Administration on Oxidative Stress

Total peroxide concentration was significantly increased in ESRD samples collected directly and at 60 min after HD compared with predialysis samples. Before the next HD session, total peroxide concentrations returned to the baseline according to the method described by Makey and Seal (45). We modified this method by using a Tris-borate-electrophoresis buffer without EDTA to avoid removal of iron from transferrin by this chelator (46,47). Serum samples were separated on 6% polyacrylamide gels that contained 6 M urea with Tris-borate-electrophoresis buffer (100 mmol/L Tris, 10 mmol/L boric acid [pH 8.4]) at 150 V (const.) for 2 h at 4°C in a Mini Protean II electrophoresis chamber (BioRad Laboratories). Blotting on nitrocellulose membrane (0.45 μm; BioRad) was performed according to the method of Plekhanov (48). Detection of transferrin on the Western blot was performed with a rabbit anti-human transferrin antibody (DAKO) as first antibody (1:500 dilution) and goat anti-rabbit HRP conjugated antibody (DAKO) as second antibody (1:7500 dilution). Then the blots were incubated with SuperSignal (Pierce), and the chemiluminescence signal was detected in a FluoroS Multimaguer (BioRad). The relative density of the bands was analyzed with the MultiAnalyst software (BioRad). The total density of the bands recognized by the anti-transferrin antibody representing apo-, holo- and monoferric transferrin was set as 100%.

Quantification of Apo-, Monoferric, and Diferric Transferrin

The routinely used method to calculate TFS from serum iron and transferrin content can give false values in serum samples that contain iv.Fe. In this study, TFS therefore was analyzed by urea-PAGE and Western blot. In this system, differently iron-loaded transferrin isoforms (apo- and holo-transferrin, plus the C- and N-terminal partially saturated monoferric transferrins) display a different electrophoretic mobility and then can be detected by immunoblot using anti-transferrin antibodies. Urea-PAGE was performed with slight modifications ac-
value. Administration of 100 mg of ferric saccharate at the end of the HD session did not further increase total peroxide concentration in the iv.Fe group (Figure 2). Peroxide generation therefore is not correlated to iv.Fe administration; the observed increase seems to result from other sources of oxidative stress related to HD.

**Effect of Iv.Fe Administration on TFS**

To obtain reliable information about TFS in the presence of iv.Fe, we used urea-PAGE to determine TFS (Figure 3). TFS significantly increased directly after iv.Fe administration and to a lesser extent in the samples collected after 1 h and returned to the baseline level before the next HD session. There was always a large amount of unsaturated transferrin present after iv.Fe treatment, whereby ~75% of total transferrin was in the only partly saturated monoferric isoform (Figure 4, A through C).

**Detection of NTBI after Iv.Fe Administration**

Although Figure 4 clearly shows that there is no “oversaturation” of transferrin in the iv.Fe patient group, NTBI could be detected in ESRD serum after iv.Fe administration by both methods used (Figure 5). In method A (Figure 5A), NTBI was mobilized with 100 mmol/L oxalate, whereas in method B (Figure 5B), the oxalate concentration was increased to 200 mmol/L, resulting in much higher maximal NTBI values. In the time-control group, NTBI remained at the baseline level during the study, whereas in the iv.Fe group, NTBI significantly increased after iv.Fe administration and remained high during the first hour. NTBI concentration returned back to the baseline level before the next HD session.

**DFO-Chelatable Iron in the iv.Fe Preparation Venofer**

Recently, Esposito *et al.* (13) assessed the presence of chelatable iron in different polymeric iron formulations directly in a fluorescence-based one-step assay with the metallosensing probes fluorescein-transferrin and fluorescein-DFO in HBS. However, the assay system used by Esposito *et al.* is limited to concentrations up to 200 μmol/L iron of the formulations, which is only a low-range approximation of the levels used therapeutically. Higher concentrations cannot be analyzed by fluorescence in a one-step assay because of inner-filter effects caused by the dark color of the iv.Fe preparations (13). As concentrations up to 600 μmol/L iv.Fe can easily be achieved in patients who receive a normal therapeutic dose in the range of 40 to 100 mg iv.Fe, it was interesting to assay the amount of chelatable iron in ferric saccharate at concentrations up to 600 μmol/L iron using a two-step assay, with removal of the colored iv.Fe preparation before the fluorescence measurement.

In the two-step assay, DFO-chelatable iron in the iv.Fe preparation Venofer could be assayed in a serial dilution with up to 600 μmol/L iron in HBS (pH 7.3). The samples were incubated with two concentrations of mobilizing reagent (method A, 100 mmol/L oxalate; method B, 200 mmol/L oxalate) in DFO-coated wells. Chelatable iron binds to DFO in the well, and after washings and therefore removal of the colored iv.Fe-preparation, iron bound to DFO was detected with the nonfluorescence iron-calcein complex, which is rendered iron-free as a result of its lower affinity than DFO. Then fluorescence of free calcein was measured (Figure 6). After incubation with 100 mmol/L oxalate (method A) for 2 h at 37°C, a mean of ~1.5% of total iv.Fe in the samples was DFO-chelatable iron; with 200 mmol/L oxalate, a mean of ~7.5% of total iv.Fe in the sample was DFO-chelatable iron. These results are similar to the results recently reported by Esposito *et al*. (13) using the one-step assay system.

**Bioavailable Iron in ESRD Serum**

In a cell culture model with HepG2 cells, the bioavailable iron from ESRD serum was assayed by the fluorescence-calcein method. Recently, we used this model to show that iv.Fe preparations transiently increased the LIP of HepG2 cells (33). Elevations of the LIP are implicated in the generation of oxidative cell injury (34). We therefore were interested in whether ESRD serum (containing *in vivo* administered iv.Fe) shows the same effect on the LIP. As dialysis treatment itself
is also likely to contribute to iron deficiency in ESRD patients, we were also interested in whether HD treatment reduces the amount of biologically available iron.

Compared with normal tissue culture conditions (control), in which the cells are relatively iron poor, the LIP was increased when the cells were incubated with serum from a healthy person (control serum; Figure 7). The LIP decreased with ESRD serum collected after HD, compared with predialysis serum, which is likely due to removal of iron by the dialysis treatment. Iv.Fe administration of 100 mg of ferric saccharate significantly increased the LIP, and the LIP returned to a lower level after 2 d (before the next HD session) but not back to the baseline predialysis value. These findings suggest that the 1-wk washout phase without iron administration (before the first HD session) could effectively reduce the bioavailable iron in ESRD serum.

Discussion

In this study, we showed a significant shift from apo- to unsaturated monoferric transferrin in HD patients who received a single infusion of 100 mg ferric saccharate during 10 min at the end of the first dialysis session, but we could not find any oversaturation of transferrin. This is in contrast to several recent reports on an apparent oversaturation of transferrin during iv.Fe therapy (14,15,38,50). One possible explanation for the opposite findings could arise from the study design: Before iv.Fe administration, our patients received no iv.Fe for 1 wk (washout phase). Moreover, these studies used routine laboratory assessments to calculate TFS from serum iron and transferrin content.
Although iv.Fe preparations are very stable polynuclear iron-(III)-hydroxide carbohydrate complexes of large mass, depending on the method used for serum iron determination, large amounts of iron from the preparations can be reduced and form complexes with iron(II)-chelators such as ferrozine. Calculated TFS in the presence of iv.Fe therefore can lead to overestimation of TFS. Urea-PAGE can be an alternative to obtain more accurate informations about TFS in the presence of iv.Fe.

One of the major concerns about increasing the efficacy of r-huEPO in ESRD patients by higher doses of iv.Fe is related to the possible generation of toxic oxygen radicals by iron (23,24). In the present study, we observed that HD significantly increased total peroxide concentration in both groups, whether they received iv.Fe or not. Peroxide generation in our ESRD patients therefore seemed not to be correlated to iv.Fe administration; the observed increase is more likely to result from other sources of oxidative stress related to HD (22).

NTBI was first described in conditions of iron overload, when TFS exceeded 100%. This led to the concept that NTBI represents a heterogeneous fraction of iron not bound to transferrin or ferritin, and it was thought to be composed of iron bound to serum albumin, citrate, and other, undefined, negatively charged ligands (29). Using this concept, iv.Fe therapy would add large amounts of NTBI to the plasma.

Iron that is not tightly bound to transferrin or other molecules can be mobilized by iron chelators such as oxalate and then can be bound by DFO (12). Using this method, we found appreciable concentrations of mobilizable "labile iron." NTBI apparently occurs despite sufficient iron-binding capacity of transferrin. The amount of detectable NTBI varied considerably, depending on the concentration of mobilizing agent used. Different concentrations of oxalate (methods A and B) apparently mobilized different pools of NTBI. Although both concentrations significantly increased NTBI after iv.Fe administration, 100 mmol/L oxalate could apparently only mobilize
that part of labile iron from the preparation that could also be chelated in vitro. However, using 200 mmol/L oxalate, the increase in NTBI concentration after iv.Fe administration was not only due to mobilizing iron from ferric saccharate, as the amount of mobilizable iron from the preparations in vitro was much lower (13).

It is interesting that with 200 mmol/L oxalate (method B), very high NTBI concentrations could be obtained in the iv.Fe group after iron infusion, but also the baseline values were much higher. This suggests that large amounts of iron from the preparations can be mobilized by 200 mmol/L oxalate. From pharmacokinetic studies in healthy volunteers, it is known that infusion of ferric saccharate leads to rapid high serum iron levels and that the mean volume of distribution of the central compartment is ~3 L, hence close to the volume of serum (37). The expected serum concentration of iv.Fe after infusion of 100 mg ferric saccharate therefore is close to 600 µmol/L serum. This means that after in vivo administration, a considerable amount of ~25% of the applied dose was mobilized by 200 mmol/L oxalate and therefore was detected as NTBI by this assay. In a solution of 600 µM iv.Fe, only 5% of total iv.Fe was DFO-chelatable iron in vitro. This means that the resting 20% of NTBI measured in the serum samples are likely to arise from another LIP. Recently, we demonstrated that parenteral iron preparations increase the intracellular LIP of the human hepatoma HepG2 cells (33). Using this model system, we show that dialysis reduced the amount of biologically available iron. This suggests that dialysis treatment itself is also likely to contribute to iron deficiency reported in ESRD patients. ESRD patients on intravenous (IV) iron therapy significantly increased the amount of bioavailable iron. Two days after iv.Fe administration, the amount of bioavailable iron decreased but remained high compared with the predialysis sample. This means that the 1-wk washout phase before the first dialysis effectively reduced bioavailable iron.

In conclusion, because preparations such as ferric saccharate do not yield iron effectively to the part of iron that is termed NTBI in vitro, processing by reticuloendothelial cells or by the liver presumably precedes iron release into the labile plasma iron pool.

Acknowledgments

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