Intravenous iron-gluconate during haemodialysis modifies plasma β2-microglobulin properties and levels

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Abstract

Background. Intravenous iron replacement therapy is routinely used for correction of anaemia in patients with end-stage renal failure. Free or labile iron, present both in parenteral iron formulations and in blood of haemodialysis (HD) patients, has the potential to induce severe oxidative processes. This study evaluated the acute in vivo effect of intravenous iron administration on the oxidation of plasma β2-microglobulin (β2m) and on its plasma levels after HD.

Methods. Iron-gluconate was administered intravenously to 14 patients receiving HD with low-flux cellulose-triacetate membranes during the first hour of the 4 h HD treatment. Each patient underwent three different dialysis treatments, during which an infusion of 62.5, 125 or 0 mg (control) of iron-gluconate was administered in random order. Plasma β2m levels and iron parameters were monitored immediately before and after each HD treatment. The molecular isoforms of β2m were studied by two-dimensional gel electrophoresis and western analysis. Levels of oxidized β2m were evaluated by reaction with 2,4-dinitrophenylhydrazine and western analysis.

Results. Both doses of iron-gluconate caused remarkable changes in the molecular properties of β2m, including shift in isoelectric point, molecular mass and degree of oxidation. Iron administration also limited the decline in plasma β2m levels to <7.5%, compared with 27.9 ± 2.7% during HD without iron.

Conclusions. Intravenous iron-gluconate led to a characteristic increase in molecular weight and in negative charge of β2m, both of which can be assumed to be consistent with reduced membrane sieving coefficients and membrane adsorption, and thus with reduced clearance of β2m.

Keywords: advanced glycation end-products; carbonyl; iron-gluconate; low flux; protein oxidation

Introduction

Intravenous (i.v.) iron replacement therapy is routinely used for correction of anaemia in patients with end-stage renal failure [1,2]. Free or labile iron, present both in parenteral iron formulations [3] and in blood of haemodialysis (HD) patients [4], has the potential to induce severe oxidative processes. Such oxidative damage is accelerated due to the pre-existing oxidative stress caused by the dialysis process per se, demonstrated by lipid peroxidation products [5] and protein oxidation products [6,7]. Iron deposits were found in joints of HD patients, in addition to β2-microglobulin (β2m) amyloids [8,9].

β2m (11.8 kDa) accumulates in plasma of uraemic patients due to impaired renal excretion, oxidative stress and inflammation, and can reach up to 92 mg/l in HD patients, while normal levels are <2 mg/l [10]. Aggregation of β2m into amyloid fibrils and their deposition in musculoskeletal tissues causes dialysis-related amyloidosis [11]. The molecular basis for β2m aggregation has been attributed to various modifications, including formation of advanced glycation end-products (AGEs) [12].

The present HD methods (such as low-flux HD, high-flux HD and haemodiafiltration) fail to normalize circulating β2m levels, although its plasma level can be lowered during each treatment [13–15]. The removal of β2m during a dialysis treatment depends on the permeability properties (molecular weight) and adsorption capacity of the dialyser membrane [14,16]. The adsorption of a given protein to the dialyser membrane, in turn, depends on the molecular properties of the protein, such as its isoelectric point (pI) [17], and on intrinsic properties of the membrane [18].
The present study was designed to evaluate the in vivo effect of i.v. iron therapy on β2m oxidation during a HD treatment and to elucidate the molecular mechanisms underlying the interactions between iron and plasma β2m.

**Subjects and methods**

**Subjects**

We studied 14 patients (eight males/six females, six diabetics) on chronic HD treatment for a mean of 33 ± 6 months, aged 72 ± 3 years (mean ± SE). All patients underwent HD treatment thrice weekly; each dialysis treatment lasted 4 h and was carried out on cellulose-triacetate hollow fibre dialysers (Nipro produced by Nissho, Osaka, Japan) using bicarbonate dialysate. All patients received maintenance i.v. iron-gluconate therapy (Ferrlecit®; Rhone-Poulenc Rorer, Cologne, Germany) and recombinant human erythropoietin treatment (4000–12 000 U/week, Recormon®; Roche Diagnostics GmbH, Mannheim, Germany). None of the patients developed hypotension or other symptoms related to the iron administration. The mean haemoglobin value was 11.2 ± 0.4 g/dl and the corresponding transferrin saturation was 28.1 ± 2.1%. The water for dialysis was compatible with the standards of the Association for the Advancement of Medical Instrumentation. Patients with evidence of infection, malignancy or severe hyperparathyroidism were excluded. Informed consent was obtained from all patients, and the institutional Helsinki committee approved the protocol.

**Study protocol**

Each patient included in the study underwent three different dialysis treatments, during which an i.v. infusion of 62.5, 125 [1,2] or 0 mg (control) of iron-gluconate (Ferrlecit®) was administered in random order. Each iron treatment was repeated twice with at least a 1 week interval between the protocols [1]. Iron-gluconate was administered during the first hour of the dialysis treatment. No other medications were administered during the treatments. For each patient, the blood flow rate and amount of ultrafiltration were the same for all studied treatments, with a mean single pool Kt/V of 1.2 ± 0.2. Blood samples were drawn from the arterial line immediately (within 10 min) before and after dialysis and assayed for iron parameters and β2m levels. Sample collection from all the patients was completed within 2 months.

**Gel filtration chromatography of plasma proteins**

All chemicals were obtained from Sigma (St Louis, MO), unless specified otherwise. Plasma samples were subjected to gel filtration so as to obtain β2m-enriched fractions: a 5 ml column of Sephacryl S-200-HR was prepared and the column was washed with 5 vols of column buffer (50 mM Tris, 100 mM NaCl, pH 7.0). A 100 μl aliquot of plasma was diluted with Tris buffer to a final concentration of 50 mM Tris at pH 7.0, centrifuged for 5 min at 12 000 g and the supernatant loaded onto the column. Proteins were eluted in column buffer in 0.5 ml fractions. The β2m-containing fractions were acetone precipitated and used for further analyses.

**Detection and quantification of carbonylated β2m**

Detection of carbonylated β2m could not be performed as previously described [6] due to the insolubility of β2m during the procedure. For the modified procedure, acetone-precipitated proteins from β2m-enriched fractions after gel filtration were resuspended in reducing Laemmli buffer. After SDS–PAGE and identification of β2m bands by western analysis, the membrane area containing the β2m bands was excised and washed briefly in a solution containing 25 mM Tris, 140 mM NaCl, 0.05% Tween-20, pH 7.4 (TBST). The membrane strip was submerged in dinitrophenylhydrazine solution (20 mM dinitrophenylhydrazine in 20% trifluoroacetic acid) for 15 min at room temperature and then neutralized in 2 M Tris solution. The membrane strip was then briefly washed in TBST and used for a second western analysis with anti-dinitrophenylhydrazine serum [6]. Untreated and in vitro oxidized commercial β2m were similarly analysed, as controls. Carbonyl signal was detected on X-ray films as described above. The intensities of carbonyl and β2m signals were quantified by densitometry using the BioCapt and Bio-Profil (Bio-1D) softwares. The ratios of carbonyl to protein signals were determined in all the samples. For each experiment, the ratio of carbonyl to protein of the ‘before dialysis’ sample was set as 100% and the ratios in all other samples were calculated relative to this sample. In the in vitro oxidation experiments, the ‘untreated’ sample was considered as 100% and the oxidized sample was expressed as a percentage of this control.

**Preparation of oxidized β2m**

Commercial β2m (from human urine) was oxidized in vitro using a metal-catalysed oxidation system of iron/ascorbate [5 mM ascorbate and 100 μM FeCl3 in phosphate-buffered saline, 5 h at 37°C] to yield a highly oxidized (carbonylated) protein. The reaction was stopped at 4°C by addition of EDTA (pH 8.0) to a final concentration of 1 mM. The oxidizing reagents were removed by acetone precipitation and the proteins were analysed by western blotting as described below.

**Gel electrophoresis and western analysis**

Plasma samples were separated by SDS–PAGE and then transferred to nitrocellulose filters. To evaluate the molecular properties and changes in β2m isoforms, proteins from five HD patients were first subjected to gel filtration chromatography, in which β2m-enriched fractions were obtained, and then separated by two-dimensional PAGE at the Smoler Proteomics Center (Faculty of Biology, Technion, Israeli Institute of Technology, Israel). The first dimensional separation was by isoelectric focusing in a pH gradient between 3 and 10 and the second dimension was molecular weight-based separation by SDS–PAGE. The proteins were then transferred to nitrocellulose filters for detection of β2m by western blot analysis with rabbit anti-human β2m (purified immunglobulin fraction, ICN Pharmaceuticals, Aurora, OH) and goat anti-rabbit–horseradish peroxidase conjugate. The chemiluminescence signal was detected on X-ray films.

**β2m and iron parameters**

Transferrin, ferritin, total iron and plasma β2m levels were determined on chemical analysers. Haemoglobin and
Iron oxidizes β₅-microglobulin and inhibits its fall during HD

haematocrit determinations were performed using a Beckmann Coulter analyser (LH 750). Transferrin saturation was calculated as $70 \times$ total iron/measured transferrin.

### Plasma variable calculations

The results of β₂m before dialysis are given as measured. In order to correct for the haemoconcentration caused by water loss during HD, the values of β₂m measured after dialysis were corrected using the correction factor $f$: $f = (1 - Hct_A)/(1 - Hct_B) \times (Hct_B/Hct_A)$ where Hctₐ and Hctᵦ are the haematocrit after and before dialysis, respectively.

### Statistics

All the results are given as the mean±SE. Paired sample $t$-test was used for analysing the changes in β₂m levels and transferrin saturation during HD. Wilcoxon signed ranks test was used for analysing the effect of the iron dose on β₂m fall, patients' age and prevailing plasma ferritin levels. Pearson correlation test was used for analysing the correlation between dialytic age and β₂m levels and between ferritin and β₂m levels.

### Results

#### Level of carbonyl groups on plasma β₂m

Iron-induced oxidation of plasma β₂m during HD was evaluated by following carbonyl groups as a marker of oxidation. For this analysis, plasma samples from six patients, before and after HD, were separated and analysed for carbonyl groups on β₂m as described above. Of the six patients examined, four patients received 125 mg and two received 62.5 mg of iron. The findings and representative western results are shown in Figure 1. After administration of 125 and 62.5 mg iron-gluconate, β₂m was composed of 51 and 22% more carbonyl groups, respectively, than β₂m obtained before HD. A significant increase of 41% in the mean carbonyl intensity was observed relative to β₂m obtained before HD and a 54% increase was observed relative to β₂m obtained after HD without iron ($n = 6$). In vitro oxidation of commercial β₂m (by metal-catalysed oxidation) similarly resulted in an increased carbonyl signal (Figure 1).

#### β₂m isoform profile

β₂m from plasma samples and the commercial sample were studied using SDS–PAGE and western analysis with anti-β₂m serum. In addition to the major 12 kDa form of β₂m, high molecular mass bands of $>100$ kDa were detected in plasma and bands of $\sim 24$ kDa appeared in the commercial sample (Figure 2A). The major form of β₂m in plasma ($\sim 12$ kDa) was further analysed and characterized.

Figure 2B shows representative results of three samples from the same patient before dialysis, after dialysis without iron and after dialysis with iron.
administration (125 mg), separated by 2D-PAGE and studied by western analysis. Before dialysis, $\beta_{2m}$ showed a pattern of several distinct isoforms, which differed in their pI values (Figure 2B). One minor isoform (no. 5) showed increased molecular mass. The pattern of $\beta_{2m}$ isoforms was conserved after HD without iron. However, this pattern changed dramatically after iron administration: the most abundant $\beta_{2m}$ spots disappeared (nos 1–4), the protein spot with a higher molecular mass became more acidic (no. 5) and a new $\beta_{2m}$ spot appeared (no. 6), with a higher molecular mass than normal $\beta_{2m}$ and with more acidic pI than the other isoforms. This form of $\beta_{2m}$ was observed uniquely after HD with iron administration. A similar $\beta_{2m}$ pattern was observed in all patients studied.

$\beta_{2m}$ isoforms profile after in vitro oxidation

The effect of in vitro oxidation on the pattern of $\beta_{2m}$ isoforms was studied in a commercial $\beta_{2m}$ preparation. The highly carbonylated protein was studied by 2D-PAGE, and $\beta_{2m}$ isoforms identified in western analysis are illustrated in Figure 3. The pattern of untreated commercial $\beta_{2m}$ differed slightly from that observed in patient plasma, evident by the presence of additional protein isoforms with very acidic pI values or increased molecular weight (Figure 3 ‘Untreated’, arrows 3–5). In vitro oxidation caused dramatic changes in the pattern of $\beta_{2m}$ isoforms, resulting in the disappearance of several isoforms (Figure 3, arrows 1, 2 and 5) and formation of several new isoforms (arrows 6–14) with more acidic (nos 6–11) or somewhat more basic (nos 12–14) pI values than the major isoform of $\beta_{2m}$. Similar changes in $\beta_{2m}$ pattern were observed after oxidation in vitro of plasma $\beta_{2m}$ (data not shown).

The effect of iron administration on plasma $\beta_{2m}$ levels

The mean level of plasma $\beta_{2m}$ before dialysis was 26.9 ± 1.4 mg/l. The baseline $\beta_{2m}$ values did not differ among different HD treatments ($P > 0.5$). Figure 4A shows the levels of $\beta_{2m}$ before and after each dialysis treatment. The mean levels of $\beta_{2m}$ decreased significantly ($P < 0.001$) from 26.2 ± 1.6 to 19.0 ± 1.4 mg/l during HD without iron. This decrease corresponded to 27.9 ± 2.7% of the initial $\beta_{2m}$ level (Figure 4B). However, no significant reduction in $\beta_{2m}$ levels was observed when iron-gluconate was administered (Figure 4A). The reduction in $\beta_{2m}$ level was blunted, such that the decrease was only < 7.5% below baseline: from 25.5 ± 1.9 to 23.4 ± 2.0 mg/l (before and after dialysis, respectively) when 62.5 mg of iron-gluconate were administered and from 28.5 ± 1.8 to 25.8 ± 2.2 mg/l for the 125 mg iron dose (Figure 4A). The blunted decrease of $\beta_{2m}$ levels after iron therapy was significantly less than the decrease during the HD without iron, irrespective of iron dosage ($P < 0.005$, Figure 4B). There was no significant difference between iron doses regarding their effect on plasma $\beta_{2m}$ levels.

Iron profile

The mean transferrin saturation value before dialysis was 28.1 ± 2.1% (Figure 5). Following iron-gluconate administration, the values after the HD treatment...
showed a significant, dose-dependent increase to 67.1 ± 4.1 and 83.8 ± 5.0% for the 62.5 and 125 mg iron dose regimens, respectively. No change was observed after dialysis without iron (28.1 ± 2.9%). Determination of transferrin saturation provided quality control for the proper execution of the study protocol. Only in two treatments with 125 mg iron was transferrin oversaturation observed.

The effect of dialytic age on $\beta_2$m levels
The correlations between $\beta_2$m levels and either dialytic age or patient age were examined. There was a correlation between dialytic age and the levels of pre-dialysis $\beta_2$m (Figure 6). In contrast, no correlation could be demonstrated between the age of the patients and the baseline levels of $\beta_2$m.

The effect of ferritin levels on $\beta_2$m decrease
The mean ferritin level before dialysis was 435 ± 82 µg/l. The effect of serum ferritin on reduction of $\beta_2$m levels during HD was examined post hoc. Patients with ferritin levels of $>300\,\mu\text{g/l}$ showed significantly ($P<0.03$) greater reduction of $\beta_2$m levels during control HD (without iron) than patients with ferritin levels of $<300\,\mu\text{g/l}$ (Figure 7A). However, reduction of $\beta_2$m levels was blunted after iron administration in both groups of patients, i.e. regardless of ferritin levels (Figure 7A).

A negative, significant correlation was found between the mean levels of ferritin and $\beta_2$m before dialysis, as shown in Figure 7B ($r = -0.64, P = 0.007$). Other iron parameters such as transferrin levels, saturation or total plasma iron did not correlate with either baseline $\beta_2$m levels or reduction of $\beta_2$m during HD.

Discussion
We have examined the effects of two clinically recommended (DOQI) iron-gluconate doses administered during a dialysis treatment on the oxidation and levels of plasma $\beta_2$m. Iron administration during HD resulted in molecular modifications of $\beta_2$m, reflected by alteration of the pI value, increased carbonyls level and changes in molecular mass, which can be assumed to be consistent with reduced membrane sieving coefficients and membrane adsorption, and thus with reduced clearance. Our study confirms earlier reports that cellulose triacetate low-flux HD is relatively inefficient, reducing circulating $\beta_2$m levels by only 30% [13–15]. Nevertheless, even this...
modest reduction in plasma β2m concentration was blunted by the administration of iron-gluconate during HD. As a result of i.v. iron, β2m levels before and after the HD treatment were virtually indistinguishable. The inhibitory effect of iron therapy on β2m reduction during dialysis was similar for 62.5 and 125 mg doses.

Based on the iron-induced changes in pI and the increase in carbonyl levels following i.v. iron, we assume that iron administration aggravated oxidation reactions pre-existing in these patients, including the oxidation of β2m. A limitation of the presented findings lies in the potential inaccuracy of quantification of protein carbonyls by densitometry. Nevertheless, to the best of our knowledge, this is the first study in vivo that i.v. iron increases the level of carbonyls on proteins such as fibrinogen [6] and albumin [7]. Our finding is in agreement with an earlier report that in vitro oxidation of β2m isoforms resulted in generation of AGE products that are manifested by an acidic shift in β2m pI [12]. The disappearance of some β2m isoforms may well be due to oxidation resulting in formation of new isoforms. Although we have not demonstrated the formation of AGE-β2m, we showed the increase in an intermediate product for AGES, namely carbonyl-modified β2m.

The iron-induced modifications of β2m were associated with a marked blunting of plasma β2m fall during a HD treatment. We imply that the iron-induced protein oxidation could account for this observation, since the passage of oxidized (AGE-modified) β2m through various dialysis membranes was previously shown to be reduced [19]. Another possible cause of reduced plasma β2m fall may be due to diminished adsorption of oxidized β2m to HD membranes, as suggested by in vitro studies [19]. The alteration of β2m pI may also impair the adsorption of β2m to the dialysis membrane [17], accounting for the reduced removal of the protein from the circulation during a dialysis with iron therapy.

To rule out the possibility that β2m synthesis or shedding during dialysis masks the fall in β2m levels, we administered iron-gluconate to chronic kidney disease patients (not on renal replacement therapy). In these experiments, iron did not cause an increase in the levels of plasma β2m measured 3 h later (data not shown). Thus, an iron-dependent increase in β2m synthesis or shading is not likely to account for our observations with HD patients.

Our findings may have some relevance to dialysis-related amyloidosis. Iron-gluconate can directly modify β2m in vivo, resulting in formation of oxidized β2m that, according to Miyata et al. [12], favours the deposition of amyloid. Secondly, as iron apparently inhibits β2m removal during HD, it may induce a slow accumulation in the levels of circulating β2m, that is strongly supported by the correlation to the overall dialytic age (Figure 6) and not to the patients age [10,20]. High concentrations of intact β2m, which have been shown to form amyloids in vitro [21], and the oxidation of β2m can lead to amyloid deposition.

Patients with high ferritin levels showed greater reduction of β2m during HD. We think that high ferritin levels minimize the concentrations of catalytically active iron in plasma, probably by its chelation, although transferrin is the accepted candidate for iron binding in the circulation. Our assumption is supported by several facts: (i) ferritin binds the toxic ferrous (Fe2+) ion, which is not bound by transferrin [22]; and (ii) in HD patients, ferritin levels are often increased and transferrin levels are decreased [6,23], thus shifting the normal ratio between these two iron-binding proteins in favour of ferritin.

Based on this study, the long-term deleterious effects of iron therapy on β2m should be explored further, particularly its effects on the onset of amyloidosis. Iron cumulative dose may be important in this regard, given that both iron-gluconate doses inhibited the decrease in β2m. Additional protocols can be addressed so as to minimize oxidation of β2m: rate and timing of iron administration and the option to use antioxidants (either free or membrane-bound) during dialysis.

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