Oxidative stress induced by iron released from transferrin in low pH peritoneal dialysis solution

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Abstract

Background. Transferrin binds extracellular iron and protects tissues from iron-induced oxidative stress. The binding of iron and transferrin is pH dependent and conventional peritoneal dialysis (PD) solutions have unphysiologically low pH values. Herein, we investigated whether conventional PD solution releases iron from transferrin and if the released iron causes oxidative stress.

Methods. Effects of PD solutions on iron binding to transferrin were examined with purified human transferrin and transferrin in dialysates drained from PD patients. Oxidative stress induced by iron released from transferrin was evaluated in terms of the formation of thiobarbituric acid reactive substance (TBARS) and protein carbonylation in the human red blood cell (RBC) membrane. The iron deposition in peritoneal tissue from PD patients was evaluated by Perls’ staining with diaminobenzidine intensification.

Results. Low pH PD solution released iron from transferrin. This iron release occurred within 1 min. Iron release was not observed in neutralized PD solution. Iron released from transferrin in low pH PD solution increased TBARS formation and protein carbonylation in the human RBC membrane. Iron deposition in peritoneal tissue from PD patients was evaluated by Perls’ staining with diaminobenzidine intensification.

Conclusions. Iron released from transferrin in low pH PD solution can produce oxidative stress in the peritoneum of a PD patient. Neutralizing PD solution can avoid this problem. Iron deposition in the peritoneum may participate in the pathogenesis of peritoneal fibrosis in PD patients.

Keywords: dialysate; fibrosis; iron; oxidative stress; peritoneal dialysis; peritoneal function; pH

Introduction

Chronic peritoneal dialysis (PD) results in peritoneal injury, which leads to a progressive reduction in dialytic efficiency. While high glucose concentration, contaminated glucose degradation products, and low pH in PD solutions have been implicated in the pathogenesis, the precise mechanisms of peritoneal injury are not well understood [1].

Iron is a metal essential to life and is necessary for a wide variety of metabolic processes, such as oxygen transport, electron transfer, nitrogen fixation and DNA synthesis. Iron exists in two oxidative states, ferrous (Fe[II]) and ferric (Fe[III]), which can donate or accept electrons. Although these redox reactions are important for biological reactions, they can also be hazardous to cells. Redox reactions involving iron play a key role in the formation of harmful free radicals that damage living cells via various pathways. To avoid the hazardous effects of iron, extracellular iron is bound by transferrin. In addition, two-thirds of serum transferrin exists as apotransferrin and will quickly capture the free iron which is released from the cell [2].

The affinity of iron for transferrin is a pH-dependent process. In plasma (pH 7.4), binding between iron and transferrin is very strong, whereas virtually no binding occurs at pH < 4.5. Iron is released from transferrin in the low pH environment of endosomes after receptor-bound transferrin is internalized into cells [2]. In the extracellular space, iron is never released from transferrin under physiological conditions [3].

Conventional PD solutions have unphysiologically low pH values, around pH 5.0. While several PD solutions that have more physiological pH values are available [4,5], low pH PD solutions are still widely used.
We report here that iron is released from transferrin in low pH PD solutions and iron released from transferrin has oxidative effects in these solutions. These effects are avoided by neutralizing the pH of the PD solution. In addition, peritoneal iron deposition was observed in PD patients.

Subjects and methods

Preparation of iron-saturated human transferrin

Iron was added to 0.2 mM human apotransferrin (Sigma, St Louis, MO, USA) in 0.1 M N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 0.025 M NaHCO3, pH 7.4, using sufficient 0.01 M ferrous ammonium sulfate in 0.01 M HCl to bring the protein to 90% saturation [6]. After a 24 h equilibration period in a 5% CO2 incubator, transferrin was also measured spectrophotometrically. Iron concentrations and unsaturated iron binding capacity (UIBC) were measured as described above.

Iron release from transferrin in low pH PD solution and oxidative stress

Red blood cell ghost membrane preparation

Heparinized blood was obtained from healthy volunteers. Red blood cells (RBC) were washed three times with PBS (pH 7.4) with removal of the buffy coat. After the addition of lysing buffer [5 mM sodium phosphate, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 4°C], the ghost membranes obtained were washed five times with lysing buffer at 4°C. Between each wash the ghost pellet was passed five times through a 22-gauge needle to promote further lysis of the cells. After contaminating red material had been removed by centrifugation (800 g, 1 min), the white ghost membranes were washed with low pH buffer (0.5 mM EDTA, 10 mM sodium acetate buffer, pH 4.0) twice and then washed with PBS (pH 7.4) three times.

Determination of oxidative stress on the RBC membrane in PD solution

Incubation was initiated by adding PD solution to the RBC membrane ghost pellet. After the incubation at 37°C for 12 h, membrane ghosts were washed three times with PBS before thioobarbituric acid reactive substance (TBARS) and protein carbonylation were determined.

To initiate the thiobarbituric acid (TBA) reaction, 60 µl of 5.4% sodium dodecyl sulphate (SDS), 300 µl of 20% acetate buffer (pH 3.5), 10 µl of 0.8% butylhydroxytoluene, 300 µl of 0.8% TBA and 35 µl of 1 mM FeCl3 were subsequently added [8]. After incubation on ice for 1 h, the reaction mixture was boiled for 1 h. TBARS was extracted with butanol and then measured by spectrophotometric assay (OD 532 nm).

Determination of protein carbonyl groups by immunoblotting

The protein carbonyl groups were determined by immunoblotting [9] using a kit (OxyBlotTM; Intergen, Purchase, NY, USA). In brief, RBC membrane protein was solubilized with SDS and the protein carbonyl group was labelled with dinitrophenylhydrazone (DNP). The protein samples were then subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking with 5% skim milk, the membrane was probed with rabbit anti-DNP antibody. After extensive washing with Tris-buffered saline (pH 7.4) with 0.005% Tween-20, the membrane was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG. Immunoreactive proteins were visualized with a chemiluminescence detection system (Pierce), followed by exposure to Kodak X-ray film. Densitometric analysis was performed using the public domain NIH ImagePC program.

Iron tissue staining

Iron depositions in peritoneal tissue samples from 12 PD patients (three autopsy specimens, nine peritoneal biopsy specimens) and four autopsy cases of haemodialysis patients who had never received CAPD therapy were examined using Perls’ reaction with diaminecobenzidine (DAB) intensification [10]. After Perls’ reaction, sections were incubated with 0.5% DAB in 0.1 M phosphate buffer at pH 7.4 for 20 min, followed by 15 min in the same medium containing 0.005% H2O2. The reaction was stopped by rinsing in deionized water.
for 30 min and then counterstained with haematoxylin. Negative control slides were prepared in the same way through the DAB intensification without the Perls' solution preincubation. No positive staining was detected in the negative control sections.

**Statistical analysis**

Data are presented as means ± SEM. Statistical significance was evaluated using the paired t-test or one-way analysis of variance (ANOVA) followed by Fisher's PLDS test. Differences at $P < 0.05$ were considered significant.

**Results**

*Conventional PD solution (pH 5.2) causes iron release from transferrin*

Dialysing purified iron-saturated human transferrin against conventional PD solution (pH 5.2) decreased the transferrin saturation rate, as shown in Figure 1A. When concentrated iron-saturated purified transferrin stock (pH 7.4) was diluted with conventional PD solution (pH 5.2), the transferrin saturation rate determined by spectrophotometric assay was also decreased, as shown in Figure 1B.

The time course of iron release from transferrin in conventional PD solution was determined by spectrophotometric assay. As shown in Figure 2, 40% of iron was released within 1 min.

**Iron release from transferrin in PD solution is pH dependent**

As shown in Figure 3, neutralizing the pH of the PD solution prevented the effects of the PD solution on iron release from transferrin. The pH of the PD solution was adjusted with NaOH or HCl. At a pH

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**Fig. 1.** Conventional PD solution (pH 5.2) causes iron release from transferrin. (A) Effect of dialysing iron-saturated purified human transferrin against conventional PD solution (pH 5.2). The transferrin saturation rate was determined using an autoanalyser method. (B) Effect of diluting iron-saturated purified human transferrin stock (pH 7.4) with conventional PD solution (pH 5.2). The transferrin saturation rate was determined by spectrophotometric assay. *$P < 0.05$.

**Fig. 2.** Iron release from transferrin occurs within 1 min in conventional PD solution (pH 5.2). The time course of iron release from transferrin was determined by spectrophotometric assay. *$P < 0.05$ vs control.

**Fig. 3.** Iron release from transferrin in PD solution is pH dependent. Neutralizing the pH of PD solution avoided the effects of the PD solution on iron release from transferrin. *$P < 0.05$ vs before the dialysis.
above 6.0, no iron release was observed. The release of iron from transferrin in low pH PD solutions was reversible, since neutralizing the pH with NaOH increased the transferrin saturation rate, compared to the control value.

Low pH PD solution caused iron release from transferrin in drained dialysates

The iron concentration and TIBC of the drain dialysate from PD patients were 1.1 ± 0.3 and 3.2 ± 0.8 μg/dl, respectively. Table 1 shows the effects of dialysing drained dialysates from the PD patients against PD solution. As described in Subjects and methods, we added 20 mM MgCl₂ to the PD solution to prevent non-specific iron binding to proteins. This addition of MgCl₂ did not affect the iron release from purified human transferrin. Dialysing the drained dialysates against a low pH PD solution decreased the transferrin saturation. This effect was not observed with neutralized PD solution. The transferrin saturation rate in serum from PD patients was also decreased by dialysing against a low pH PD solution containing 20 mM MgCl₂ (data not shown).

Iron released from transferrin in low pH PD solution produces oxidative stress on membrane lipids and proteins

To investigate the effects of iron released from transferrin on oxidative stress on biological materials, we incubated human RBC membranes in PD solution. When iron-saturated transferrin (100 μg iron/dl) was added to the PD solution at pH 5.2, the production of TBARS was markedly enhanced, as shown in Figure 4. This effect of transferrin was abolished by addition of the iron chelator, deferoxamine (2 mM). When neutralized PD solution was used, no effects of iron-saturated transferrin were observed.

Western analysis of the protein carbonyl content of the RBC membrane showed the protein carbonyl content to be markedly increased by the addition of iron-saturated transferrin to the PD solution (pH 5.2). Figure 5 presents the densitometric analysis of protein carbonylation (molecular weight between 100 and 30 kDa). This effect was also abolished by deferoxamine. Iron-saturated transferrin had no effect in PD solution at pH 7.0.

Iron deposition in the peritoneum of PD patients

We examined peritoneal iron deposition in PD patients using Perls’ staining with DAB intensification. In all peritoneal samples we examined, some form of iron deposition was observed. In several cases, iron deposition was prominent around the fibrotic area facing the peritoneal cavity (Figure 6A). Iron deposition was also

<table>
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<tr>
<th>pH of PD solution</th>
<th>Transferrin saturation before dialysis (%)</th>
<th>Transferrin saturation after dialysis (%)</th>
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<tbody>
<tr>
<td>pH 5.2</td>
<td>37.6 ± 4.7</td>
<td>17.8 ± 2.6</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>37.6 ± 4.7</td>
<td>35.1 ± 5.2</td>
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</tbody>
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Data are means ± SEM.  
*P < 0.05 vs before dialysis.

Fig. 4. Iron released from transferrin in low pH PD solution exerts oxidative stress on membrane lipids; TBA reaction. Addition of iron-saturated transferrin (TF) to the PD solution at pH 5.2 enhanced the TBARS production in human RBC membranes. This effect of transferrin was abolished by the addition of iron chelator deferoxamine (DEF). When neutralized PD solution (pH 7.0) was used, the effect of iron-saturated transferrin was not observed. *P < 0.05 vs control.

Fig. 5. Iron released from transferrin in low pH PD solution exerts oxidative stress on membrane proteins; protein carbonylation. The protein carbonyl content was markedly increased by the addition of iron-saturated transferrin (TF) to the PD solution (pH 5.2). This effect of transferrin was abolished by the addition of deferoxamine (DEF). When neutralized PD solution (pH 7) was used, this effect of iron-saturated transferrin was not observed. *P < 0.05 vs control.
observed in some vascular walls (B). In two cases of sclerosing encapsulating peritonitis, iron deposition was extensive (C and D). In the autopsy specimens from four haemodialysis patients that lacked CAPD-associated morphological changes, iron deposition was not observed.

**Discussion**

Our results clearly show that iron is released from transferrin in conventional low pH PD solution and that the released iron exerts oxidative stress. Drained dialysates from PD patients contain transferrin, which releases iron in low pH PD solution. Since the iron release occurs within 1 min, it can be concluded that iron is released from transferrin in some portion of the residual PD solution after new PD solution is introduced into the peritoneal cavity, before the neutralization of PD solution occurs. Low pH PD solution may decrease pH in the interstitial space of the peritoneum and cause iron release. Iron deposition was observed in all peritoneal samples from PD patients, and iron deposition was prominent in the fibrotic area facing the peritoneal cavity. While the origin of this iron cannot be determined, the iron deposition may be attributable to the iron released from transferrin.

Various degrees of diffuse peritoneal fibrosis have been documented in the patients who have been on long-term PD [1]. Contributions of oxidative stress to tissue injury and fibrosis have been shown in a variety of experiments [11]. We showed that iron released from transferrin in low pH PD solution exerts strong oxidative stress. Although the concentration of iron-saturated transferrin in peritoneal fluid is lower than the concentration we used in these *in vitro* experiments, the peritoneum of PD patients is exposed to the iron-induced oxidative stress whenever new low pH PD solutions are introduced into the peritoneal cavity. Since various oxidative reactions are irreversible, the iron-induced oxidative damage accumulates in the peritoneum of patients receiving long-term PD with low pH PD solutions.

We found iron deposition in the fibrotic peritoneum of PD patients and extensive iron deposition was observed in the severely fibrotic peritoneum of patients with sclerosing encapsulating peritonitis. Excess iron deposited in the liver has been shown to cause hepatic fibrosis, through oxidative stress, in several animal models [12]. In addition, intraperitoneal administration of a high dose of iron dextran reportedly causes peritoneal fibrosis in rat PD models [13]. These observations support the hypothesis that oxidative stress induced by iron released from transferrin in low pH PD solution may play an important role in peritoneal injury and fibrosis in PD patients.

Several immunohistochemical studies have shown that advanced glycation end products (AGEs) accumulate in the peritoneal tissue of PD patients [14]. This suggests that a high concentration of glucose results in local generation of AGEs in the PD patient peritoneum. It has been shown that oxidative stress plays a key role in the formation of AGEs. Since metal-catalysed oxidation accelerates the formation of AGEs [15], iron released from transferrin in low pH PD solution may contribute to peritoneal AGEs formation in PD patients.

For the treatment of anaemia in dialysis patients, the use of intravenous iron is widely recommended [16]. Recently, several studies in haemodialysis patients have shown intravenous iron to induce oxidative stress [17], which may accelerate vascular complications. Iron excess may also enhance the pre-existing
risk for infectious complications or malignant disorders in dialysis patients [18,19]. In addition to these proposed hazardous effects of iron treatment in dialysis patients, our results raise the possibility that iron excess may enhance the peritoneal injury in PD patients.

Neutralizing the pH of PD solution, i.e. raising the pH above 6.0, prevents iron release from transferrin and also avoids the oxidative stress produced by iron-saturated transferrin. Since a low pH environment reportedly enhances the oxidative stress induced by iron [20], a low pH PD solution may enhance the oxidative stress induced by iron deposited in tissues as we observed in peritoneal tissues. This possible oxidative stress can also be avoided by neutralizing the PD solution.

Recently, several PD solutions with a neutral pH value have been introduced. Several markers of peritoneal injury in drained dialysates are reportedly improved by switching from conventional PD solution to the neutral PD solutions [4,5]. While these neutral PD solutions also have the advantage of lower concentrations of cytotoxic glucose degradation products, the iron-related mechanisms we proposed herein may also contribute to the improved biocompatibility of these solutions. While the use of a neutral PD solution for 2 years does not improve the peritoneal transport characteristics [4], a longer study period may reveal some benefits of neutral PD solution in peritoneal transport parameters. It is also possible that neutralized PD solution would decrease the incidence of sclerosing encapsulating peritonitis, which is a potentially fatal complication of PD.

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References


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