N-ACETYLCYSTEINE MODULATES EFFECT OF THE IRON ISOMALTOSIDE ON PERITONEAL MESOTHELIUMAL CELLS

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Intravenous (i.v.) iron supplementation is used in patients on chronic peritoneal dialysis (pd). Iron induced intraperitoneal inflammation observed in our previous studies with iron sucrose may deteriorate the function of the peritoneum as the dialysis membrane. We evaluated effect iron compound, iron-isomaltoside-100 (IIS) on the peritoneal mesothelial cells (MC). We studied the effect of iv treatment with IIS ± N-acetylcysteine (NAC) on the dialysate parameters and function of MC. In 7 uremic pd patients IIS 200 mg was infused i.v. ± NAC 600 mg. Afterward, a 4 hours exchange was performed with Dianeal 1.5%. As a control dialysate exchange preceding IIS treatment was used. Inflammatory parameters of the drained dialysates as well as the dialysates and IIS effects on MC were evaluated in ex vivo experiments. Intravenous infusion of IIS resulted in an increase of the dialysate Fe (+147%, P < 0.01). Concentrations of the dialysates inflammatory mediators were increased: interleukin-6 (IL-6) +39%, P < 0.02, monocyte chemoattractant protein-1(MCP1) +50%, P < 0.02, and hyaluronan (HA) +64%, P < 0.02. Simultaneous i.v. infusion of NAC prevented increase of the dialysate inflammatory mediators. Dialysates collected after IIS treatment induced oxidative stress in MC (+29%, P < 0.05) and stimulated IL-6 synthesis (+64%, P < 0.05) in MC; no such effect was seen in dialysates obtained after simultaneous IIS and NAC iv. treatment. IIS used as the additive to culture medium stimulated synthesis in MC of IL6 (+76%, P < 0.001) and plasminogen activator inhibitor-1 (PAI-1) (28%, P < 0.001) whereas synthesis of tissue plasminogen activator (t-PA) was reduced (~16%, P < 0.001). These changes were prevented in the presence of NAC 1 mmol/L. Intravenous administration of IIS results in the mild stimulation of intraperitoneal inflammation. IIS changes MC phenotype to the inflammatory one with reduced fibrinolytic activity. These effects are prevented by NAC.

Key words: iron isomaltoside, peritoneal dialysis, mesothelium, inflammation, N-acetylcysteine, interleukin-6, monocyte chemoattractant protein, hyaluronan

INTRAVENTION

Iron supplementation is a standard procedure in uremic patients on renal replacement therapy. In most patients it is performed through intravenous (i.v.) infusions of iron formulations, which is increasingly regarded as efficient and safe treatment and more practical, because it requires less frequent patient visits to the hospital (1). The significant effectiveness of such therapy for correction of iron deficit in renal patients has been described in large clinical studies, although results from various centers still show potential side effects of that treatment (2-4). In peritoneal dialysis patients intravenous iron supplementation is considered as the second-line treatment in patients who respond poorly to oral iron supplementation or who have high iron requirements (5). The effectiveness of ferric carboxymaltose or iron sucrose in the correction of iron deficit in peritoneal dialysis patients was recently reported, but no information is provided about the potential adverse effects of such treatment on the peritoneum (6, 7). Previously, we found that i.v. infusion of iron sucrose causes intraperitoneal inflammation, and spent dialysate was cytotoxic towards mesothelial cells (8).

Intravenously infused iron-containing complexes, are ingested by the resident phagocytes in the liver, spleen, and bone marrow. After degradation of their carbohydrate shell, iron is released into the bloodstream and linked with iron-transporting protein such as transferrin. The situation is less clear and probably more complicated in patients treated with peritoneal dialysis. Iron compounds infused intravenously can simultaneously diffuse into the peritoneal cavity, where after ingestion by macrophages, free iron can be released. Transferrin diffusing into the peritoneal cavity, in the presence of the low pH dialysis fluid, releases free iron, which causes oxidative stress (9). Oxidative stress is the main factor initiating the cytotoxic effect of iron (10). Therefore simultaneous infusion of iron compounds and antioxidants may reduce the oxidative stress in the peritoneum. N-acetylcysteine (NAC), which was used in the present study, is a precursor for synthesis of glutathione, being one of the most potent antioxidant compounds. Protective effects of NAC were described in various experimental models in which oxidative stress was present, such as inflammation in the pulmonary epithelial cells (11) or colitis in rats (12).

Supplementation of peritoneal dialysis patients with NAC resulted in various positive effects such as preservation of the
residual renal function (13) or reduction of the systemic interleukin 6 (IL-6) level (14). On the other hand, Agarwal et al. found increased oxidative stress in uremic patients treated with iron sucrose, which was followed by proteinuria and tubular damage, but simultaneous application of NAC did not prevent these changes (15). In another study, iron sucrose did not induce oxidative stress in peritoneal dialysis patients (16). These data show a large variation in the systemic effects of iron compounds used in uremic patients.

We present results from the study in which we evaluated the effect of NAC administered intravenously in patients treated with i.v. IIS on the inflammatory parameters of the dialysate and its effect on the mesothelial cells in in vitro culture. Previously, we found significant peritoneal toxicity of iron sucrose (8). There are no clinical data showing negative effects of IIS on the peritoneal membrane but there are also no data about the effect of that iron formulation on peritoneal mesothelial cells.

**MATERIAL AND METHODS**

The study was performed in 7 male uremic patients on CAPD, who were treated with IIS (Monover®) as an iron supplement. The mean age of the patients was 52.7 ± 8.4 years, and the duration of the renal replacement therapy was 32.3 ± 9.2 months. No episodes of peritonitis were reported for three months prior to enrolment. Iron supplementation was administered as iron-isomaltoside-100 (IIS) in a dose of 200 mg given i.v. at four week intervals (dose I and dose II). The second dose of IIS was preceded by i.v. infusion of NAC in a dose of 600 mg. Before and after infusion of IIS, 4 hours of dialysis exchanges were performed with Dianea 1.5%. Both dialyse effluents were collected, spun down, and frozen at –86°C for further analysis. None of the patients reported any side effects during i.v. treatment with IIS.

Dialysates concentrations of IL-6, monocyte chemoattractant protein-1 (MCP1), and hyaluronan (HA) were measured with standard ELISA kits (R&D, MN, USA). The concentration of total iron in the dialysates was measured with a colorimetric kit from Alpha Diagnostics (Warsaw, Poland). The effect of the collected dialysates on human peritoneal mesothelial cells in in vitro culture was evaluated during subsequent experiments.

**Experiments on mesothelial cells**

Experiments were performed on primary cultures of human peritoneal mesothelial cells obtained from effluent dialysates from patients treated with CAPD and stored in the Cells Bank in the Department of Pathophysiology. Cells were collected from the effluent dialysates during the first six months of treatment with peritoneal dialysis.

Cells were grown in the standard culture medium M199 supplemented with hydrocortisone (1 μg/ml), antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) and 10% fetal calf serum (FCS). Experiments were performed on the mesothelial monolayers in 48-well plates.

**Intracellular generation of free radicals**

MC monolayers were exposed to the studied dialysates for 24 hours. Afterwards the intracellular generation of reactive oxygen species (ROS) was evaluated with a 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) probe. Cells were labelled with a probe for 45 minutes at 37°C and afterwards lysed with the lysis buffer (Promega, Madison, USA). The fluorescence of the cells lysates was measured in a fluorimeter, using the wavelengths 485 nm for excitation and 535 nm for emission. The data are expressed in arbitrary units expressed per μg cells protein.

**Secretory activity of the cells exposed to the dialysates**

MC monolayers were exposed to the studied dialysates for 24 hours. At the end of the incubation, dialysates were replaced in all groups with medium M199 + 10% FCS and the second 24 hours were used to assess the secretory activity of the cells. At the end of the second incubation, supernatants were collected for the measurement of IL-6 and MCP-1. Synthesis of these molecules was expressed per number of the cells in the well.

**Effect of N-acetylcysteine on the iron-isomaltoside-100 effect on mesothelial cells**

MC monolayers from 8 donors were exposed to the culture medium (control group), medium with IIS 15 μg/dL, or medium with IIS additionally supplemented with NAC1 mmol/L. Concentration of IIS used in the in vitro experiments was chosen based on the results of the dialysate iron concentration after i.v. infusion of IIS. After 24 hours’ incubation generation of free radicals was evaluated in the studied cells, as described above, and their secretory activity was reflected by supernantant concentration of IL-6, tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1). Concentrations of these compounds were measured with the standard ELISA methods (R&D, MN, USA) and expressed per number of the cells in each well.

**Analysis of genes expression**

Genes expression was studied on the mesothelial monolayers in 6-well plates, which were exposed, analogically as in the secretory experiments, during 24 hours to control medium, medium with IIS 15 μg/dL or medium with IIS 15 μg/dL and NAC 1 mmol/L. At the end of the incubation, total RNA was isolated using the ReliaPrepTM RNA Cell Miniprep System (Promega, USA) treated with DNase I using DNA-free DNase Treatment and Removal Reagent (Ambion). RNA was reverse-transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). Relative levels of mRNA of 3 genes: IL-6 [F:ATGAACCTCTTCTCCACAAGC; R:GGTTTCTGCGATGCCTGTTT], t-PA [F:CAAGCCAGGAAATCCATGCC; R:GCCATGATCGTGGTGCCTG], PAI-1 [F:GTCCTGTGAATGCTCCCTACT; R:CGGTCATTCCCAAGTCTCTA] were studied in triplicate from each experiment and normalized to the levels of an internal house-keeping gene: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [F:TTCTGTCATGCAAGTGAAAC; R:GATGATGTTCGAGAGGCC]. Relative gene expression was calculated using the 2−ΔΔCt method (17).

**Statistical analysis**

Results are presented as mean values ± SD. Statistical analysis was performed with the Friedman test with the post hoc Kruskal Wallis multiple comparison test. A P-value of less than 0.05 was considered significant.

**RESULTS**

Intravenous infusion of IIS in a dose of 200 mg resulted in a significant increase of the iron concentration in the dialysate. Levels of iron in the dialysates before infusion of IIS were...
comparable: 6.2 ± 2.9 µg/dL in the dose I group and 6.5 ± 3.3 in the dose II group. Intravenous infusion of IIS resulted in a significant increase of the dialysates’ iron concentrations: 15.5 ± 1.2 µg/dL in dose I (P < 0.01 versus preinfusion dialysate) and 15.8 ± 2.2 µg/dL in dose II (P < 0.01 versus preinfusion dialysate). The IIS induced increase of the iron concentration was similar in patients without NAC pretreatment and with NAC infusion.

Intravenous administration of IIS resulted in an increased concentration of IL-6, MCP-1 and HA in the dialysate, and these effects were significantly weaker in cases when the i.v. infusion of IIS was preceded by an infusion of NAC (Fig. 1). Exposure of the mesothelial cells in conditions of the in vitro culture to the tested dialysates obtained after IIC treatment without simultaneous application of NAC resulted in the increased intracellular generation of free radicals (+29%, P < 0.05) and synthesis of IL-6 (+64%, P < 0.05). Dialysates collected after the simultaneous IIS and NAC i.v. infusion did not stimulate either intracellular oxidative stress or release of IL-6 from the mesothelial cells (Fig. 2).

IIS studied at the highest concentration of that drug observed in the drained dialysates changed the functional properties of the mesothelial cells in in vitro culture, and these effects were modified by NAC. Intracellular generation of free radicals was increased in the presence of IIS by 90% versus control, P < 0.001, and that effect was not present in cells simultaneously exposed to NAC (Fig. 3A). NAC prevented the IIS increase of IL-6 and PAI-1 synthesis in the mesothelial cells (Fig. 3B and 3D), and at the same time, inhibited the IIS induced decrease of t-PA synthesis in these cells (Fig. 3C). Changes in the secretory activity of the mesothelial cells were reflected in changes of the genes expression (Fig. 4). The ratio of t-PA/PAI-1 levels reflects the fibrinolytic activity of the mesothelial cells and it was reduced in the presence of IIS: 0.084 ± 0.025 versus 0.133 ± 0.026.

**Fig. 1.** Dialysates concentrations of (A) interleukin 6 (IL-6), (B) monocyte chemoattractant protein-1 (MCP1) and (C) hyaluronan (HA) obtained before and after treatment with iron-isomaltosied-100 (IIS) ± N-acetylcysteine (NAC); (n = 7).
DISCUSSION

The results of the present study suggest that intravenous infusion of IIS changes the properties of the dialysate in uremic patients treated with chronic peritoneal dialysis. During the relatively short exchange of the dialysis fluid, lasting only 4 hours, the concentration of iron in the dialysate was higher by more than two times as compared to the control group. At the same time an enhanced intraperitoneal inflammatory reaction was observed, reflected by the increased dialysate levels of IL-6, MCP-1, and HA (Fig. 1). Iron suppresses the respiratory burst of the peritoneal leukocytes (18), which suggests that the observed intraperitoneal inflammation was due to the stimulation of the mesothelial cells (Fig. 2).

Results from the in vitro experiments indicate that the inflammatory reaction in the mesothelial cells is due to oxidative stress (Fig. 2A), and that may explain the beneficial effect of the antioxidant NAC in that scenario. In patients infused with IIS and NAC, dialysate inflammation was similar to that in the control group (Fig. 1). We measured iron concentration in the dialysate, but not its specific molecular forms (i.e. bound to transferrin or free) in which it was present in the peritoneal cavity. Therefore, we cannot say whether reduced intraperitoneal inflammation was due only to NAC antioxidant activity or due to different forms of iron present in the dialysate. Results from the in vitro experiments confirm that the presence of NAC reduces the iron-induced inflammatory phenotype of the mesothelial cells. Due to its small molecular size (m.w. 163), comparable to glucose, NAC infused i.v. rapidly diffuses into the peritoneal cavity, and enhances the antioxidant properties of the dialysate. Previously, it was found that NAC can prevent AGE glucose induced glycation in the peritoneum (19) and oxidative stress in mesothelial cells exposed to dialysis fluid (20). Our results show that NAC can also defend the peritoneal mesothelium against iron toxicity due to its antioxidant action. Previously we found that sulodexide reduced the dialysate induced functional changes in the peritoneal mesothelial cells and its effect was also linked with suppression of the intracellular oxidative stress (21).

We found that IIS used in a concentration similar to the dialysate iron levels obtained after i.v. infusion of that drug, reduced, in vitro conditions, the fibrinolytic activity of the mesothelial cells due to suppression of t-PA synthesis and stimulation of PAI-1 production (Fig. 3). This change is typical for intraperitoneal inflammation (22). Impaired dialysate
Fibrinolysis results in the peritoneal deposition of fibrin, which may initiate the epithelial-to-mesenchymal transition of peritoneal mesothelial cells leading to peritoneal fibrosis (23). We found that NAC can inhibit the negative effect of iron in the peritoneal mesothelium restoring the normal t-PA/PAI-1 ratio (Fig. 3). An animal study showed the protective action of NAC.

Fig. 3. Intracellular generation of (A) free radicals, (B) synthesis of interleukin-6 (IL-6), (C) tissue-plasminogen activator (t-PA) and (D) plasminogen activator inhibitor-1 (PAI-1) in mesothelial cells exposed in vitro to iron-isomaltoside-100 (IIS) 15 µg/dL ± N-acetylcysteine (NAC), 1 mmol/L; (n = 8).

Fig. 4. Expression of genes regulating synthesis of interleukin 6 (IL-6), tissue plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1) after exposure of these cells to culture medium supplemented with iron-isomaltoside-100 (IIS) 15 µg/dL ± N-acetylcysteine (NAC), 1 mmol/L. Results are presented as % of control, which is the expression of genes in cells exposed to the standard medium.
against the formation of adhesions after abdominal surgery (24). On the other hand, in a rat model of chlorhexidine-induced encapsulating peritoneal sclerosis the application of NAC resulted in a reduction of peritoneal inflammation and neovascularization, but not fibrosis (25). In all studies, NAC reduced the morphological changes in the peritoneum induced by inflammation.

Proinflammatory effects of IIS within the dialysate dwelling in the peritoneal cavity may not be strong enough to cause side effects such as *i.e.* abdominal pain. That may explain why in clinical studies evaluating the efficiency of intravenous iron supplementation in peritoneal dialysis patients, no side effects are reported (6, 7, 26, 27). In our study, no patient reported any side effect during i.v. infusion of IIS, but increased dialysate inflammatory reaction was present in all of them. In the clinical scenario the potential injurious effect of IIS to the peritoneum may be less significant because such therapy is used approximately once, at one month intervals. On the other hand, the impact of IIS within the peritoneal mesothelial cells may be prolonged due to the accumulation of iron within these cells (8). However, we did not find any difference in the dialysate IL6, MCP-1 and HA levels, in the control exchanges, before IIS treatments, performed at 4-week interval. Perhaps the cumulative effect of IIS on mesothelial cells will start to appear after several months of treatment. We found that already after 4 hours of exposure of the mesothelial cells in *in vitro* culture to IIS present in the medium, iron content within these cells was increased and persisted during the following hours when the cells were incubated in iron-free medium (data not shown). That means that we can expect the accumulation of the toxic effect of iron in the peritoneal mesothelium as early as after their single exposure to that ion. Therefore, one can expect that prolonged, even mild, decreased fibrinolytic activity of peritoneal mesothelial cells may predispose subjects to deposition of fibrin within the peritoneum, which can contribute to the morphological changes of the peritoneum observed in patients on chronic peritoneal dialysis (28). Further studies are required to confirm this hypothesis. We must take into account the fact that intraperitoneal inflammation induced by the process of peritoneal dialysis is different in individual patients. That means that patients with strong dialysis induced intraperitoneal inflammation may be more prone to the negative effect of iron on the mesothelial cells. The observations presented in this paper are not unique to IIS, and we previously observed similar, but even mild, decreased fibrinolytic activity of peritoneal pulmonary epithelial cells.

Intravenous iron supplementation is necessary for peritoneal dialysis patients with poor intestinal absorption of that ion or its severe systemic deficiency. Our results show that NAC can diminish the potentially harmful effects of such therapy on mesothelial cells. However further experimental and clinical studies are necessary to confirm that hypothesis.

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