Apoptotic stress pathway activation mediated by iron on endothelial cells in vitro

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Abstract

Background. Iron sucrose (Fe-S) and low-molecular-weight iron dextran (Fe-D) have been used successfully in the treatment of anaemia in chronic kidney disease patients. However, some side effects, such as endothelial cell dysfunction have been reported. Mechanisms by which iron can induce endothelial cell damage have not been completely understood. This study was designed to examine the effect of Fe-S and Fe-D on bovine aortic endothelial cells in vitro.

Methods. Cell proliferation was determined by [3H] thymidine incorporation, cytotoxicity by lactate dehydrogenase, pro-Caspase-3 by immunoblotting; and Caspase-3 activity using a colorimetric assay. Expression of the apoptosis stress pathway proteins Bcl-2 and Bax and cycle arrest proteins p53 and p21\textsuperscript{WAF/CIP1} were examined by immunoblot. Cell apoptosis was tested by terminal deoxynucleotidyltransferase-mediated nick-end labeling (TUNEL) and DNA fragmentation.

Results. Both iron preparations inhibited cell proliferation. This effect was more important and occurred at lower concentrations in Fe-S than Fe-D cultured cells. Expression of p53 and p21\textsuperscript{WAF/CIP1} increased in cells incubated with Fe-S, but not with Fe-D. Bcl-2 expression was significantly down-regulated in cells incubated with Fe-S in comparison with Fe-D, while Bax expression was not modified by the iron compounds. Pro-Caspase-3 expression and Caspase-3 activity increased only in cells treated with Fe-S. Apoptosis was present in cells treated with Fe-S.

Conclusions. Our results demonstrate that Fe-S exerts a greater inhibitory effect on endothelial cell proliferation than Fe-D. The mechanisms involved in this process may be related, at least in part, to over expression of proteins related to the cell cycle arrest and apoptosis stress pathway.

Keywords: apoptosis; endothelial cells; intracellular signal; iron

Introduction

Prescribed iron has become an essential therapy in the management of anaemia in advanced chronic kidney disease. In fact, intravenous iron alone can raise the haematocrit levels without the use of recombinant human erythropoietin (rHu-EPO). At the same time, intravenous iron administration is simple and assures rapid and continuous therapeutic iron levels when compared with oral iron.

An important interest in the long-term side effects of iron administration has been generated recently. It has been suggested that iron may have a deleterious effect on vascular endothelium [1,2]. Numerous investigations have demonstrated the association between high iron storage levels and atherosclerotic heart disease [3,4]. Similarly, there is experimental evidence supporting the potential toxic effect of iron on the vascular endothelium [5,6].

One of the most investigated mechanisms by which iron may induce atherosclerosis is through the production of potent oxidant species, such as low-density lipoproteins (LDL) and iron hydroxical radical [7]. Oxidized LDL has cytotoxic capacity that can promote endothelial cell damage and potentially induce the accumulation of monocytes with further progression of the atherosclerotic lesion [8].

There is other evidence implicating the potential toxic effect on endothelial cells.

Duffy et al. [9] using deferoxamine, a potent iron chelator, in patients with coronary atherosclerosis demonstrated a significant enhancement in nitric-oxide-mediated endothelium-dependent vasodilation. Their results suggest that iron availability contributes to impaired nitric oxide action in atherosclerosis. More recently, Zager et al. [10] demonstrated that iron can inhibit endothelial cells proliferation in vitro.
The mechanisms by which iron may induce endothelial cell cytotoxicity are not well-known. Therefore, in the present study we examined the possible role of iron in the apoptosis of endothelial cells. In brief, apoptosis could be mediated by two mechanisms, one being extrinsic and the other intrinsic [11]. The extrinsic pathway comprises the surface receptor CD95 and the transfer of the apoptotic signal from CD95 to Bcl-2 by Caspase-8. The intrinsic pathway, or stress-induced apoptosis, embraces a spectrum of extracellular stimuli, such as radiation and drugs, and intracellular stimuli, which includes DNA damage, oxidative stress, and protein misfolding. In the stress-induced apoptosis, Bcl-2 proteins seem to be essential for the activation of the apoptotic signal to the mitochondria [12]. Cytochrome c is released from the mitochondria in response to Bcl-2 [13]. Cytosolic cytochrome c induces the formation of multiprotein complex, apoptosome, leading to the activation of Caspase-3, with the final execution of the apoptosis process [12].

The present investigation was carried out to examine the effects of two iron compounds, low-molecular-weight iron dextran (Fe-D) and iron sucrose (Fe-S), on in vitro endothelial cell proliferation. We measured the effect of both iron preparations on proteins involved in the stress-induced apoptosis pathway, such as Bcl-2, Bax and Caspase-3. Additionally, we evaluated two cyclin-dependent kinases (CDK), p53 and p21WAF/CIP1, as early markers of cell-growth arrest.

**Material and methods**

**Cell culture**

Bovine aortic endothelial cells (BAECs) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) at the fourth passage and used between passages 5 and 12. Cells were grown in DMEM-F12 (Sigma Chemicals, St Louis, MO, USA) containing 10% certified fetal bovine serum (FBS) (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA), L-glutamine (2 mM), penicillin (100 U/ml)/streptomycin (100 µg/ml) and incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The day before cell treatment the medium was replaced with DMEM-F12 containing 2% FBS for 8–16 h. Cells were then incubated under three different conditions: control cells incubated with 2% FBS, cells treated with different Fe-S concentrations of elemental Fe from 0 to 1 mg/dl (0, 0.01, 0.1 and 1 mg/ml) in 2% FBS.

**Cytotoxicity assay and pH determination**

Lactate dehydrogenase (LDH) released from BAECs was measured as an index of iron-induced cytotoxicity. Cells were incubated for 24 h using similar concentrations of Fe-S and Fe-D as described above. The percentage of LDH released to the cultured medium at each time period represents an index of membrane cellular integrity. LDH was assayed using a colorimetric LDH assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI, USA) according to the manufacturer’s specifications. Absorbance was measured at 490 nm. The amount of LDH present in the supernatant was expressed as a percentage of the total LDH of the cultured cells. Incubations media pH was determined in all experiments before and after incubation time.

**Immunoblot analysis**

Bcl-2, Bax, Pro-Caspase-3, p53 and p21WAF/CIP1, were evaluated by immunoblot from BAEC total lysates incubated overnight with both iron compounds at increasing concentrations of elemental Fe from 0 to 1 mg/dl (0, 0.01, 0.1 and 1 mg/ml) in 2% FBS. After treatment with the different iron concentrations, BAECs were washed with ice-cold phosphate-buffered saline and resuspended in 100 µl of RIPA buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Igepal, 0.5% sodium deoxycholate, 0.5% SDS, 100 mM NaCl, 100 mM sodium fluoride) with 1 mM sodium orthovanadate, 10 mM aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 0.01 mg/ml PMSF-like protease and phosphatase inhibitors and then harvested by scraping. Cells were homogenized by passing the suspension through a 25-gauge needle and placed on ice for 30 min. The cell extracts were centrifuged at 10 000 g for 20 min at 4°C. Protein was determined by the Bradford method and 25 µg were used for the western blot analysis. Proteins were resolved on polyacrylamide gels (12% for Caspase-3 and p53, and 17% for p21WAF/CIP1, Bcl-2 and Bax) and blotted onto polyvinylidene difluoride membranes. Non-specific binding was blocked with 5% fat-free milk in TBST [50 mM Tris-HCL (pH 7.5) 0.15 NaCl, 0.05% Tween-20] for 1 h at room temperature. Filters were incubated in the presence of (i) a monoclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), (ii) a polyclonal rabbit anti-p21WAF/CIP1 (Santa Cruz Biotechnology) to study cell cycle regulators, (iii) an anti-Caspase-3 polyclonal antibody (Santa Cruz Biotechnology) to evaluate the total and active Caspase-3, (iv), a hamster anti-human Bcl-2 antibody (Pharimingen, San Diego, CA, USA) and (v) a rabbit anti-Bax antibody (Santa Cruz Biotechnology) to assay the integrity of the mitochondria. All incubations were performed in TBS, 3% skim milk for 2 h at room temperature or overnight at 4°C. Membranes were then washed three times for 5 min each with TBST buffer and incubated with the horseradish peroxidase-conjugated appropriated secondary antibody (1:5000) (Santa Cruz Biotechnology).
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Biotecnology) for 1.5h at room temperature. Finally, immune complexes were detected by enhanced chemiluminescence ‘West Pico’ reagent (PIERCE Biotecnology, Rockford, IL, USA). The intensity of the bands was evaluated using the ‘Quantity One’ software in a G-800 Densitometer (Bio-Rad, Hercules, CA, USA).

Caspase-3 activity

BAECs were seeded in 12-well plates and incubated overnight in the presence of the iron compounds. Cells were then washed twice with D-PBS. Caspase-3 activity was determined by a colorimetric CaspACE Assay System (Promega) using 50 uM Z-VAD-FMK (a cell-permeable pan-caspase inhibitor) and DEVD-pNA (acetyl Asp-Glu-Val-Asp p-nitroanilide) as substrate. Lysed cells were centrifuged at 15000g for 20 min at 4°C. The supernatant was collected and Caspase-3 specific activity was detected by measuring the proteolytic cleavage of the substrate DEVD-pNa for 4 h at 37°C to pNa (p-nitroaniline). Absorbance was measured at 405 nm. Protein content of the cell extracts was determined by Bradford’s method. Caspase specific activity (SA) was expressed as: SA= 100pmole pNa liberated per h/μg protein.

DNA fragmentation

BAEC were incubated overnight with Fe-D or Fe-S at the final iron concentrations of 0, 0.01, 0.1 and 1 mg/ml, respectively. Adherent and floating cells were harvested and lysed with a hypotonic lysis buffer (10 mM Tris-HCL, 10 ml EDTA, 1% SDS, 0.5% Triton X-100). After incubation at 4°C for 15 min, lysates were then incubated with 10μl of 10 mg/ml RNase-A (Sigma) for 1 h at 37°C and followed by an overnight incubation with a 10 μl of 20 mg/ml Proteinase K (Life Technologies) at 40°C. DNA was extracted using the chloroform: phenol: isopropanol mixture method. DNA was then precipitated overnight with isopropanol at −70°C and washed with chilled 95% ethanol. DNA electrophoresis was performed at a constant voltage of 100 V in horizontal 2% agarose gels. DNA bands from the apoptotic cells were detected by fluorescence microscopy.

TUNEL assay

BAEC cells were grown in 10cm Petri dishes and fixed for 15 min in 1% paraformaldehyde in PBS pH 7.4 and incubated under the different experimental conditions, as outlined in the preceding text. Apoptosis in situ was determined using a terminal TUNEL assay (ApoTag. Serologicals Corporation, GA). Additionally, samples were stained with propidium iodide (0.5 ug/ml) as counterstain for total DNA content in the mounting medium and examined by fluorescent microscopy using standard fluorescein excitation and emission filters (520 nm).

Statistical analysis

Results from all experiments are expressed as mean ± standard deviation (SD). Multiple comparisons of the mean values were analysed by one-way ANOVA with the post hoc Bonferroni test. Other comparisons of the means were performed by unpaired Student’s t-test.

Results

Endothelial cell proliferation

Cell growth was analysed by [3H] thymidine incorporation assay. Figure 1A and B show [3H] thymidine incorporation by BAECs after 24 and 48 h incubation with different Fe-S and Fe-D concentrations. Cells incubated in the absence of iron were used as controls. After 24 h, Fe-D did not affect cell growth. However, Fe-S at the concentrations of 0.5 and 1mg/ml significantly decreased BAECs proliferation as compared with controls (P < 0.01 and P < 0.001, respectively) (Figure 1A). In Figure 1B, the 48 h incubation demonstrated that Fe-D tended to decrease BAECs proliferation but not significantly, whereas, Fe-S inhibited cell growth even at the concentration of 0.01 mg/ml (P < 0.05).

Lactate dehydrogenase (LDH) and pH determination

To assess whether the iron compounds have a potential cytotoxic effect on BAECs that could explain the decrease in BAECs proliferation, LDH release in the supernatant media was determined. As shown in Figure 2, at the iron concentrations of 0.01 and 0.1mg/ml, there were no differences in the LDH release from cells treated with either Fe-S or Fe-D or the controls. However, cells treated with 1mg/ml of Fe-S, demonstrated a significant increase in LDH.

Fig. 1. Effect of iron sucrose (Fe-S) and low-molecular-weight iron dextran (Fe-D) on bovine artery endothelial cell proliferation (BAEC). Cell growth was analysed by [3H] thymidine incorporation assay. (A) BAEC were incubated for 24h in the absence (control) or presence of different iron concentrations of Fe-S and Fe-D (0.01, 0.1, 0.5 and 1 mg/ml). (B) BAEC were incubated for 48h in the absence (control) or presence of different iron concentrations of Fe-S and Fe-D (0.01, 0.1, 0.5 and 1 mg/ml). Results were analysed by ANOVA and expressed as mean ± SD of each experimental condition from four separate experiments performed by triplicate. *Fe-S vs Control (P < 0.01), **Fe-S vs Control (P < 0.001), ***Fe-S vs Control (P < 0.001).
release compared with Fe-D and controls \((P < 0.001)\). Incubation media pH levels determined before and after incubation were stable and did not affect cell viability.

**Expression of p53 and p21^{WAF\text{-}CIP1}\**

To investigate other mechanisms that could be involved in cell-growth inhibition, we examined the role of apoptosis and studied the CDK, an early marker of cell-growth arrest. BAECs cells were synchronized by serum deprivation for 12 h. As shown in Figure 3A, Fe-S increased p53 expression by 71 to 93\%, compared with Fe-D \((P < 0.03\) and \(P < 0.001\), respectively). Similarly, Fe-S up-regulated p21^{WAF\text{-}CIP1} expression by 53 to 94\% when compared with Fe-D \((P < 0.05\) and \(P < 0.001\), respectively) (Figure 3B).

**Bcl-2 and Bax expression\**

Mitochondria membrane integrity was assessed by measuring the relative expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins. Figure 4A shows a dose-dependent reduction in Bcl-2 expression by 58, 63 and 98\% in Fe-D treated cells, compared with Fe-S \((P < 0.01)\). Meanwhile, there were no differences in Bax expression between Fe-D and Fe-S (Figure 4B).

**Pro-Caspase-3 and Caspase-3 expression and activity\**

Caspase-3 is a protease that can be activated in the initial stage of apoptosis. Therefore, in the next series of experiments the expression of both the inactive Pro-Caspase-3 protein (37 kDa) and the active Caspase-3 (20 kDa), were examined. When BAECs were treated with either iron form at 0.01, 0.1 or 1 mg/ml, Fe-S induced an increased Pro-Caspase-3 expression by 61, 80 and 89\%, when compared with Fe-D (Figure 5A). Likewise, at the same iron doses, the activated Caspase-3 expression was markedly increased, by 56 to 83\%, in Fe-S compared with the Fe-D-treated cells \((P < 0.05)\).

To confirm the expression of Caspase-3, we evaluated the activity of this protein by a colorimetric assay. As shown in Figure 5B, Fe-S concentrations of 0.01 and 0.1 mg/ml produced a significant increase in Caspase-3 activity compared with control \((P < 0.01\) and \(P < 0.005\), respectively). In contrast, at all concentrations, Fe-D did not modify Caspase-3 activity when compared with controls. When a very low iron concentration of 0.001 mg/ml was used, only
Fe-S treated cells exhibited a significant increase in Caspase-3 activity. When the effects of the two iron forms were compared, differences in Caspase-3 activity were obtained only at the concentration of 0.1 mg/ml \((P < 0.001)\).

DNA fragmentation

DNA nuclear fragmentation is considered one of the morphological changes observed in apoptotic cells. Figure 6 displays the absence of DNA fragmentation in cultured BAECs with the different concentrations of Fe-D. In contrast, a ‘DNA ladder’ was present in cells treated with 0.1 and 1 mg/ml of Fe-S.

TUNEL

A TUNEL assay was performed using experimental and control cells. Figure 7 shows the immunofluorescent microphotography of adherent cells. The percent of apoptotic cells (green divided by the number of red cells per field) present in the control cells was 1.7% (Figure 7A). A 5 and 11% increase in apoptosis were observed when cells were treated with 0.1 and 1 mg of Fe-D, respectively (Figure 7B and C). However, incubation with the same concentrations of Fe-S produced a 20.4 and 38.3% increase in the apoptotic rate (Figure 7D and E).

Discussion

The present study demonstrates that Fe-S induces a marked decrease in BAECs proliferation when compared with Fe-D treated cells. In addition, Fe-S causes more endothelial cell dysfunction than Fe-D. This effect was associated with activation of the apoptosis stress pathway and cell cycle regulating proteins.

These findings are in agreement with a recent report by Zager et al. [10] demonstrating that Fe-S inhibits aortic endothelial cell proliferation and showed more cytotoxicity than other iron compounds. The pathophysiological mechanism implicated in these effects
included mitochondrial dysfunction induced by the iron compounds.

Our study provides additional information regarding the iron-induced alterations in the sub-cellular signalling and cell cycle in endothelial cells in vitro. It is known that CDK coordinates cells throughout the cell cycle and that it is regulated at checkpoints by inhibitors, such as p53 and p21WAF/CIP1. Similarly, these two proteins have been implicated in cell-growth blockage, leading to cell-cycle arrest [14]. In fact, p21WAF/CIP1 has been shown to be transcriptionally up-regulated by p53 in response to DNA damage and hypoxia, leading to inhibition of retinoblastoma protein phosphorylation and cell-cycle arrest at G1 to S transition [15]. In the present study, we have found that Fe-S induces CDK inhibitors p21WAF/CIP1 and p51 expression more than Fe-D. Additionally, when BAECs were incubated with Fe-S, the anti-apoptotic Bcl-2 was down-regulated, whereas Fe-D cultured cells did not show changes. This suppression could lead to the alteration of mitochondrial permeability, resulting in the release of Cytochrome c to the cytosol, which represents an irreversible step to cellular apoptosis. Thus, Fe-S increases two CDK inhibitors, down-regulates Bcl-2, and initiates the activation of the apoptotic process. Interestingly, the pre-apoptotic Bax expression was not affected by either iron compounds, indicating that Bcl-2 could act independently of Bax.

The activation of diverse caspases is a subsequent event in the stress-mediated apoptosis and intimately related to the liberation of Cytochrome c from the mitochondria [16]. Therefore, we further examined the possible role of the effector Caspase-3 in the apoptosis process by measuring the expression of the inactive form of Pro-Caspase-3, as well as the active Caspase-3. Our results suggest that both proteins were up-regulated by Fe-S-treated cells, but not in Fe-D-treated cells. Similar findings have been described in previous studies. Indeed, activation of Caspase-3 with a decrease in Bcl-2 expression and no change in Bax levels occurred during the endothelial cell apoptosis [17]. Finally, as a last step in the apoptosis process, we have identified that only Fe-S was able to produce DNA fragmentation, indicating that Fe-S has more cytotoxic effect than Fe-D.

The mechanisms by which iron has a deleterious effect on endothelial cells is under investigation. As mentioned previously, Zager et al. [10] suggested that mitochondrial dysfunction was responsible for the cytotoxic effect associated with iron compounds. More recently, Agarwal et al. [18] found in patients with a glomerular filtration rate of <60 ml/min that intravenous Fe-S administration produced a rapid increase in oxidative stress, which was associated to transient proteinuria and tubular damage. These reports reflect the complex biochemical events carried out by the iron preparation on the cells, which could include the loss of mitochondria integrity and the production of oxidative stress. Regardless of the mechanisms whereby iron may induce cell damage, there is unquestionable new evidence suggesting the potential cytotoxicity of Fe-S when compared with other intravenous iron compounds [10,18,19].

All these observations could explain the endothelial-cell dysfunction observed in some iron-treated patients. In fact, the effect of iron on endothelial cells and the potential risk of cardiovascular diseases have been recently reported in patients with increased iron storage. Elevation of serum ferritin as an indicator of iron overload was linked to atherosclerotic lesions. In patients with serum ferritin levels of 200 µg/L or greater, the risk of myocardial infarction almost doubled when compared with patients with lower ferritin levels [20]. In another study of 79 haemodialysis patients receiving hydroxide sucrose iron, Dru¨eke et al. [2] reported a correlation between increased carotid artery intima media thickness and cumulative iron doses.

In conclusion, the results of the present study demonstrate that Fe-S could potentially activate the CDK inhibitors p53 and p21WAF/CIP1, increase the expression of Bel-2, up-regulate Caspase-3 and, finally, induce DNA breakdown. All these findings may explain the mechanisms involved in the inhibition of BAECs proliferation produced by iron sucrose, inducing cell cycle arrest and apoptosis. These in vitro results cannot necessarily be extrapolated to the clinical setting and the controversy regarding the high-molecular-weight iron dextran formulation’s risks, namely anaphylaxis, remain to be clarified. Until further studies are conducted, caution should be exercised with decisions on iron prescriptions.

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