Original Paper

Deferoxamine Promotes MDA-MB-231 Cell Migration and Invasion through Increased ROS-Dependent HIF-1 α Accumulation

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Key Words

Deferoxamine • Hypoxia-inducible factor-1 α • Reactive oxygen species • Extracellular signal-regulated kinase 1/2 • Breast cancer

Abstract

Background/Aim: Deferoxamine (DFO), an iron chelator, has been reported to induce hypoxia-inducible factor-1 α (HIF-1 α) expression. HIF-1 α plays a critical role in promoting tumor metastasis. However, the molecular mechanisms underlying induction of HIF-1 α in breast cancer cells remain unknown. Our aim was to ascertain whether DFO enhanced cancer metastasis in MDA-MB-231 cells. **Methods:** Cellular reactive oxygen species (ROS) was measured by flow cytometry. Cell migration was determined by wound healing and transwell assays. Protein and mRNA expression were detected by western blotting and RT-PCR, respectively. **Results:** DFO treatment enhanced cell migration and invasion, while HIF-1 α expression was significantly up-regulated at the post-transcriptional level. However, treatment with a NADPH oxidase inhibitor, diphenyleneiodonium (DPI), strongly inhibited ROS generation and HIF-1 α expression, as well as cell migration and invasion. Notably, DFO treatment increased extracellular signal-regulated kinase (ERK)1/2 phosphorylation. Inhibition of ROS production with DPI attenuated DFO-induced ERK1/2 activation. Moreover, a MEK1 inhibitor, PD98059, suppressed DFO-induced cell migration and invasion. **Conclusion:** DFO-induced HIF-1 α expression involves a cascade of signaling events including ROS generation, activation of ERK signaling, and subsequent promotion of cell migration and invasion. These findings indicate a risk associated with DFO and other iron chelators for treatment of tumors with invasive potential.

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Introduction

Iron (Fe) is an essential element for cellular functions including cell proliferation. Because of their rapid proliferation, tumor cells have an increased requirement for Fe [1]. Recent studies indicate that excess accumulation of Fe may be a risk factor for breast cancer [2, 3]. Accordingly, breast cancer cells display an abnormal Fe metabolism. For example, breast cancer cells express high levels of transferrin receptor 1 and internalize Fe from transferrin at a very high rate [4, 5]. In addition, expression of ferritin, transferrin receptors, transferrin, and iron-regulatory proteins 1 and 2 are substantially elevated in breast cancer cells with an aggressive mesenchymal phenotype. These observations suggest that Fe imbalance is associated with the progression of breast cancer toward a more advanced malignant phenotype [6]. Thus, targeting cancer with chelating agents in an attempt to alter cellular Fe homeostasis or metabolism is a promising treatment approach [7, 8].

Fe chelation has been examined both *in vitro* and *in vivo* as a therapeutic intervention for breast cancer [9–11]. Deferoxamine (DFO) was the first Fe chelator to be examined for antitumor effects. It primarily causes a reduction in total body Fe by chelating extracellular Fe. However, Fe depletion by DFO has been shown to increase hypoxia-inducible factor-1 alpha (HIF-1 α) expression [12, 13]. HIF-1 α is a critical mediator of the physiological response to hypoxia, and its dysregulation promotes tumor angiogenesis and metastasis [14]. Moreover, primary breast cancer biopsies have revealed that high expression of HIF-1 α is associated with significantly increased risks of treatment failure, disease relapse, distant metastasis, and patient mortality [15–17]. Thus, clinical Fe chelation therapy may have unintended effects including promotion of tumor metastasis by increasing HIF-1 α expression.

Reactive oxygen species (ROS) has been implicated in the aggressive behavior of cancer cells, but its role in the regulation of HIF-1 α expression appears to be controversial. Increased HIF-1 α expression has been found to contribute to mitochondrial activity and specifically ROS formation during hypoxia [18–20]. More importantly, ROS derived from NADPH oxidases can be produced in the cytosol [21, 22]. In addition, it plays a larger role in HIF-1 α expression during normoxia than hypoxia [23]. Under normoxic conditions, PI-3K/Akt and MEK1/extracellular signal-regulated kinase (ERK) pathways modulate HIF-1 activation by basic fibroblast growth factor (bFGF) in human breast cancer cells [24]. Conversely, other studies have demonstrated a decrease in HIF-1 α expression with increasing ROS [25, 26]. Furthermore, some studies have shown no effects on mitochondrial ROS [27]. These contrasting results suggest that it is important to focus on the relationship between HIF-1 α and ROS in breast cancer.

We hypothesized that DFO could promote breast cancer cell migration and invasion through accumulation of HIF-1 α , which contributes to the metastatic process. In the present study, we investigated the effects of DFO on cell migration and invasion in a breast cancer cell line. We also characterized the association between the treatment and ROS activity, as well as the expression of HIF-1 α and downstream genes. Furthermore, we explored the role of the ERK1/2 pathway in DFO-induced cell migration and invasion *in vitro*.

Materials and Methods

Cell culture

All the experiments were performed in a biology laboratory of the Institute of Disease Control and Prevention of People's Liberation Army (Beijing, China). The human breast cancer cell line MDA-MB-231 was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO₂.

Cytotoxicity assay

Cells (4×10^4) were seeded in 96-well culture plates and allowed to adhere overnight at 37 °C. The cells were then incubated in medium (100 μ L) containing 30 or 300 μ M DFO. Control cells were not exposed to DFO. After 24 hours of incubation, the growth medium was exchanged with 100 μ L MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent in DMEM, and the cells were incubated for 3 hours at 37 °C. Cells viability was analyzed by measuring the absorbance at 570 nm with a microplate reader.

ROS detection

Cellular ROS was measured by flow cytometry using H_2DCF -DA as a fluorescent probe. The cells were incubated with 10 μ M H_2DCF -DA for 30 minutes at 37 °C, washed, resuspended in phosphate-buffered saline, and then analyzed by a FACSort (BD Biosciences, San Jose, CA, USA). The mean fluorescence intensity was quantified by CellQuest software (BD Biosciences, San Jose, CA, USA).

Wound healing assay

MDA-MB-231 cells were seeded in 6-well plates and cultured to 80–90% confluence. The monolayer was then wounded by dragging a plastic pipette tip across the surface. Then, the cells were incubated with medium containing 30 or 300 DFO, 300 μ M DFO + 100 μ M diphenyleneiodonium (DPI), or 300 μ M DFO + 100 μ M PD98059. The control cells were not exposed to DFO. After 24 hours of incubation, phase contrast images of the wounds were recorded, and cells that had migrated into the wounded areas were counted to quantify the cell migration.

Transwell invasion assay

Migration of MDA-MB-231 cells was determined using transwell chambers (24-well plates, 8- μ m pore size, Corning). Cells (1×10^5) were loaded into the upper chamber containing medium with 30 or 300 μ M DFO, 300 μ M DFO + 100 μ M DPI, or 300 μ M DFO + 100 μ M PD98059. After 24 hours of incubation, the cells were fixed and stained with 0.1% crystal violet. The number of cells that migrated to the underside of the membrane was counted under a light microscope. Three independent experiments were carried out in triplicate.

Semi-quantitative RT-PCR

Total RNA from the cells was isolated using Trizol (Invitrogen, CA, USA). Reverse transcription was performed with a PrimeScript® II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The primer sequences (Invitrogen, Shanghai, China) were as follows: HIF-1 α , (forward) 5'-GCACAGGCCACATTCACG-3' and (reverse) 5'-TGAAGATTCAACCGGTTTAAGGA-3'; vascular endothelial growth factor (VEGF), (forward) 5'-TGCCCGCTGCTGTCTAAT-3' and (reverse) 5'-TCTCCGCTCTGAGCAAGG-3'; 3-phosphoinositide-dependent protein kinase-1 (PDK1), (forward) 5'-TGAAGTACCTTGCACAT-3' and (reverse) 5'-TGAAGCAGCACTGAACACG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (forward) 5'-AAGGTCATCCCTGAGCTGAA-3' and (reverse) 5'-CCCCTCTTCAAGGGGTCTAC-3'. PCR was carried out in a 25 μ L volume including 1 $\times$ PCR buffer, 50 ng cDNA, 25 pmol each of forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 3 U Taq DNA polymerase. Amplification conditions were denaturation at 95 °C for 5 minutes, 25 cycles of 95 °C for 60 seconds, 66 °C for 60 seconds, and 72 °C for 60 seconds, followed by a final 10-minute extension at 72 °C. GAPDH was used as an internal control. RT-PCR products were analyzed by electrophoresis on 1.2% agarose gels stained with ethidium bromide.

Western blot analysis

Cells were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche, Switzerland). Total protein (10 μ g) was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred onto nitrocellulose membranes. The blots were blocked using 5% blocking reagent in Tris-buffered saline with Tween-20 (TBS-T) for 1 hour at room temperature and then incubated with a primary antibody (1:1000) overnight at 4 °C. After washing with TBS-T, the blots were incubated with a secondary antibody conjugated to horseradish peroxidase (1:1000) for 1 hour at 37 °C. After extensive washing, the complexes were visualized using a West Pico chemiluminescent kit (Pierce, Rockford, IL, USA).

Fig. 1. DFO treatment enhances the migration and invasion of MDA-MB-231 cells. MDA-MB-231 cells were cultured without or with 30 or 300 μ M DFO, and then the effect of DFO on cell viability was determined by (A) MTT assays. Results are the mean $\pm$ SD from three independent experiments (* $p < 0.05$ compared with untreated cells). (B) Wound healing migration assay of DFO-treated and untreated MDA-MB-231 cells. Distances migrated by DFO-treated and untreated cells were compared in three independent experiments. (C) Invasiveness of MDA-MB-231 cells (treated with or without DFO) was quantified by transwell assays. Results are the mean $\pm$ SD value of three independent experiments (* $p < 0.05$ compared with untreated cells).

Statistical analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Differences between means were determined by one-way analysis of variance followed by a least significant difference test for multiple comparisons. A p -value of less than 0.05 was regarded as statistically significant.

Results

DFO promotes cell migration and invasion

Compared with untreated cells, the cell viability was not significantly affected by 30 or 300 μ M DFO treatment for 24 hours (Fig. 1A). Morphological observation also indicated that 30 or 300 μ M DFO exhibited no cytotoxicity in MDA-MB-231 cells (data not shown). Thus, the above concentrations and an incubation time of 24 hours were applied in the following experiments. Wound healing and transwell assays were used to assess the effects of DFO on MDA-MB-231 cell migration and invasion. As shown in Fig. 1B, treatment with 30 or 300 μ M DFO significantly enhanced cell migration in wound healing assays (Fig. 1B). In transwell assays, the results showed a significant increase of cell invasion by treatment with DFO. Compared with untreated cells, 30 and 300 μ M DFO enhanced MDA-MB-231 cell invasion by

Fig. 2. DFO increases the expression levels of HIF-1 α protein, VEGF mRNA, and PDK1 mRNA in MDA-MB-231 cells. MDA-MB-231 cells were cultured without or with 30 or 300 μ M DFO. (A) Representative blot and electrophoretogram of HIF-1 α protein and mRNA, respectively. Compared with untreated cells, DFO-treated cells showed higher

levels of HIF-1 α protein but not HIF-1 α mRNA. (B) Representative levels of VEGF and PDK1 mRNAs. Compared with untreated cells, VEGF and PDK1 mRNA levels were increased in DFO-treated cells.

24 and 129%, respectively (Fig. 1C). These data indicate that 30 and 300 μ M DFO promotes cell migration and invasion, but has no significant cytotoxicity in MDA-MB-231 cells.

DFO elevates HIF-1 α protein expression via a post-transcriptional mechanism

To explore the effects of DFO on HIF-1 α expression, MDA-MB-231 cells were treated with 30 and 300 μ M DFO. After treatment, HIF-1 α expression was detected by western blot analysis. The results showed up-regulation of HIF-1 α protein expression in cells treated with 30 or 300 μ M DFO for 24 hours (Fig. 2A). Conversely, treatment with 30 or 300 μ M DFO had no obvious effect on HIF-1 α mRNA expression (Fig. 2A). Researches of others also demonstrate that Fe depletion by DFO increase HIF-1 α expression *in vivo* and *in vitro* [12, 13, 28]. HIF-1 α transactivates multiple categories of genes to promote tumor growth, including VEGF, one of the most potent angiogenic factors, and PDK1, a metabolic enzyme that converts tumor metabolism to glycolysis by inactivating a tricarboxylic acid cycle enzyme, pyruvate dehydrogenase. To further investigate whether DFO-induced HIF-1 α could up-regulate VEGF and PDK1, we determined their mRNA expression by RT-PCR after 24 hours of DFO treatment (Fig. 2B). As a result, treatment with DFO significantly increased mRNA expression of VEGF and PDK1 in MDA-MB-231 cells, indicating that DFO regulates HIF-1 α expression at the post-transcriptional level.

NADPH oxidase-derived ROS regulates HIF-1 α -dependent cell migration and invasion

We also examined whether ROS plays a role in DFO-induced up-regulation of HIF-1 α in MDA-MB-231 cells. Flow cytometric analysis using the fluorescent dye H₂DCF-DA demonstrated that DFO increased the cellular fluorescence intensity compared with that in control cells. Treatment of MDA-MB-231 cells with 30 or 300 μ M DFO increased cellular ROS generation by 28 and 61%, respectively. The elevated ROS levels were consistent with HIF-1 α up-regulation (Fig. 3A). It has been reported that ROS produced from NADPH oxidase is responsible for HIF-1 α stabilization under hypoxic conditions [29]. Treatment of cells with the NADPH oxidase inhibitor DPI (100 μ M) significantly inhibited cellular ROS generation (Fig. 3B) and attenuated HIF-1 α up-regulation induced by 300 μ M DFO (Fig. 3C). These results suggest that ROS is responsible for DFO-induced up-regulation of HIF-1 α . Accordingly, wound migration assays showed that treatment of the cells with 100 μ M DPI resulted in a decrease of DFO-induced migratory activity compared with that in DFO-treated cells (Fig. 3D). Similarly, transwell invasion assays indicated a decrease of DFO-induced invasiveness by treatment with DPI compared with that in DFO-treated cells (Fig. 3E). These results strongly indicate that induction of NADPH oxidase activity and subsequent augmentation of ROS generation by DFO treatment might activate HIF-1 α -dependent cell migration and invasion.

Fig. 3. NADPH oxidase-derived ROS promotes aggressive cellular behaviors by up-regulating HIF-1 α expression. MDA-MB-231 cells were cultured without or with 30 or 300 μ M DFO, or 300 μ M DFO + 100 μ M DPI. (A) DFO treatment of the cells increased ROS generation. The mean fluorescence intensity of MDA-MB-231 cells (with or without DFO treatment) was quantified by flow cytometry. Results are the mean $\pm$ SD of three independent experiments (* $p < 0.05$ compared with untreated cells). (B) DPI treatment (100 μ M) effectively attenuated DFO-induced ROS activity. The mean fluorescence intensity of the cells was quantified by flow cytometry. Results are the mean $\pm$ SD of three independent experiments (* $p < 0.05$ compared with untreated cells). (C) DPI treatment inhibited DFO-induced HIF-1 α up-regulation. (D) Wound healing assays showed that cells treated with 100 μ M DPI exhibited a decrease in DFO-induced migratory activity compared with that of DFO-treated cells. (E) Transwell assays showed a decrease in DFO-induced invasiveness by treatment with DPI compared with that of DFO-treated cells. Results are the mean $\pm$ SD of three independent experiments (* $p < 0.05$ compared with untreated cells; # $p < 0.05$ compared with DFO-treated cells).

DFO-induced cell migration and invasion is mediated via the ERK pathway

Because NADPH oxidase 1 transcription is enhanced by the ERK pathway [30], we investigated whether DFO regulates NADPH oxidase through the ERK pathway. Western

Fig. 4. ERK1/2 pathway mediates DFO-induced cell migration and invasion. MDA-MB-231 cells were cultured without or with 300 μ M DFO, 300 μ M DFO + 100 μ M DPI, or 300 μ M DFO + 100 μ M PD98059. (A) ERK1/2 phosphorylation was increased in DFO-treated cells compared with that in untreated cells. Treatment with 100 μ M DPI down-regulated ERK1/2 activation to near basal levels. (B) Wound healing assays showed that cells treated with 100 μ M PD98059 exhibited a decrease in DFO-induced migratory activity compared with that of DFO-treated cells. (C) Transwell assays showed a decrease in DFO-induced invasiveness by treatment with PD98059 compared with that of DFO-treated cells. Results are the mean $\pm$ SD of three independent experiments (* $p < 0.05$ compared with untreated cells; # $p < 0.05$ compared with DFO-treated cells).

blotting demonstrated that the level of ERK1/2 phosphorylation was aberrantly stimulated in DFO-treated cells (Fig. 4A). Rathnasamy et al. also reported that administration of DFO to hypoxic microglial cells enhanced p-ERK phosphorylation [31]. Treatment with 100 μ M DPI significantly attenuated DFO-induced ERK1/2 activation to near basal levels (Fig. 4A). There were no changes in the levels of total ERK1/2 following treatment with DFO or DPI (Fig. 4A). Furthermore, wound migration assays showed that PD98059 treatment resulted in a decrease of migratory activity compared with that in DFO-treated cells (Fig. 4B). Similarly, transwell invasion assays indicated a decrease of cell invasiveness compared with that in DFO-treated cells (Fig. 4C). These findings indicate that production of NADPH oxidase-derived ROS is mediated by the ERK signaling pathway in response to DFO treatment.

Discussion

The purpose of this study was to investigate the potential effects of DFO on breast cancer cell migration and invasion *in vitro* and its possible regulatory mechanism. The results showed that DFO enhanced cancer migration and invasion *in vitro* and NADPH oxidase-derived ROS was essential for HIF-1 α -dependent migration and invasion induced by DFO, which were mediated by ERK1/2 signaling pathways.

Considering the enhanced dependence on Fe metabolism in cancer cells, previous studies have attempted to use Fe chelators as a therapeutic intervention for breast cancer [9-11]. As a Fe chelator, DFO is usually beneficial and shows preventive effects related primarily

to its selective high affinity for Fe^{3+} and binding in a hexadentate fashion. However, studies have shown that DFO induces HIF-1 α accumulation even under normoxia [32, 33]. Clinical evidence indicates that HIF-1 α is associated with poor prognoses of patients with invasive breast carcinoma [34]. In this study, we examined the effect of DFO on cell migration and invasion, and found a positive effect on these functions. Results also demonstrated that DFO dramatically regulated HIF-1 α expression in MDA-MB-231 cells at the post-transcriptional level, which was involved in DFO-induced cell migration and invasion. Moreover, DFO promoted cell migration and invasion through up-regulation of VEGF and PDK1. These results provide direct evidence that DFO has practical effects on the migration and invasion of breast cancer cells by up-regulation of HIF-1 α expression.

HIF-1 α plays a critical role in the promotion of tumor angiogenesis and metastasis. However, the molecular mechanisms underlying the induction of HIF-1 α in tumor cells are unclear. Recently, a number of studies have suggested that mitochondrial ROS is involved in the stabilization and activation of HIF under hypoxic conditions [35]. Moreover, increased ROS and ROS-dependent stabilization of HIF under conditions of normal oxygen tension have been reported in cancer cells [36]. DFO is frequently used as a hypoxic-mimetic agent, which has been shown to mimic the effects of oxygen deprivation by inducing a number of hypoxia-response genes [37]. Notably, DFO may increase the production of hydroxyl radicals and exert pro-oxidant and cytotoxic effects [38-40]. These findings show that ROS is mediated through up-regulation of HIF-1 α . Consistent with these reports, our results clearly suggested that increased ROS production was required for HIF-1 α stabilization induced by DFO, and these effects were attenuated by a NADPH oxidase inhibitor. We also revealed that high levels of ROS production resulted in enhanced cell migration and invasion. Conversely, a decrease of ROS activity induced by the NADPH oxidase inhibitor suppressed cell migration and invasion. Taken together, the present study suggests that DFO treatment of breast cancer cells with high ROS production promotes breast cancer metastasis through up-regulation of HIF-1 α expression.

Elevated ROS generation is associated with alterations of metastatic genes in malignant breast cancer cell lines [41]. Previous studies show that ROS can activate downstream PI3K/AKT and ERK1/2 pathways that regulate HIF-1 and VEGF expression [42, 43]. Shi et al. reported that bFGF induces HIF-1 α activation and VEGF release in the T47D breast cancer cell line, involving PI3K/Akt and MEK1/ERK pathways [24]. Furthermore, ERK1/2 translocation to the nucleus to exert part of its biological activity is promoted by hypoxia [44]. Phosphorylation of p300 by ERK increases HIF-1 α transcriptional activity by increasing formation of the HIF-1/p300 complex [45]. In the present study, DFO treatment up-regulated ERK1/2 phosphorylation in the breast cancer cell line MDA-MB-231. To further test whether DFO-induced ERK1/2 activation specifically regulated cell migration and invasion, we used PD98059, a specific inhibitor of the upstream ERK regulator MEK1, to suppress the ERK1/2 pathway. After treatment with the cell, 100 μM PD98059 was sufficient to down-regulate ERK1/2 activation to basal levels without nonspecific effects (data not shown). Furthermore, reduced phosphorylation of ERK1/2 was observed after inhibition of ROS production, which correlated with cell migration and invasion. Thus, these results suggest a dependency of ROS on ERK1/2 signaling pathways and HIF-1 α expression in MDA-MB-231 cells. Accordingly, it may be reasonable to speculate that DFO-induced enhancement of HIF-1 α protein stabilization is in part mediated through the ERK1/2 pathway.

In conclusion, the present study strengthens the hypothesis that Fe chelation with DFO may have the unintended effect of promoting breast cancer metastasis by increasing HIF-1 α expression. Our results show that DFO significantly enhances breast cancer cell migration and invasion *in vitro*. Furthermore, NADPH oxidase-derived ROS is essential for DFO-induced cell migration and invasion through up-regulation of HIF-1 α expression. It is very likely that ROS mediates stabilization of HIF-1 α protein through the ERK1/2 pathway. Based on these findings, we propose that accumulation of HIF-1 α is a critical step for DFO-induced cell migration and invasion, which may potentially lead to metastasis and recurrence of breast cancer.

Disclosure Statement

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