A Mycobacterial Iron Chelator, Desferri-Exochelin, Induces Hypoxia-inducible Factors 1 and 2, NIP3, and Vascular Endothelial Growth Factor in Cancer Cell Lines

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ABSTRACT

Hypoxia is a key phenomenon in tumor behavior, selecting for resistance to apoptosis, conferring resistance to radiotherapy and chemotherapy, and also inducing angiogenic factors such as vascular endothelial growth factor (VEGF). Exochelins are naturally evolved iron chelators produced by Mycobacterium tuberculosis. Because iron chelation has been reported to activate the hypoxia-inducible factor (HIF), we investigated the effects of an exochelin [desferri-exochelin (DFE) 772SM] on this hypoxia-inducible pathway and downstream target genes. DFE induced HIF-1α and HIF-2α transcription factors regulating the hypoxic response in the breast tumor cell line MDA468. DFE was 10 times more potent and more rapid in onset of effect than the clinically used iron chelator deferoxamine. The expression of downstream hypoxia-responsive target genes VEGF and the proangiogenic protein NIP3 was activated by transcription. MDA468 proliferation was inhibited via HIF-independent pathways, related to other effects of iron chelation. DFE inhibited effects of VEGF on endothelial cell proliferation. DFE potentially could be useful in cancer therapy by inducing apoptosis via NIP3 in conjunction with other non-HIF-related growth inhibitory pathways and blocking endothelial proliferation despite the presence of VEGF.

INTRODUCTION

Mammalian cells adapt to a hypoxic environment by increased expression of specific hypoxia-inducible genes. A key player in this repertoire of responses is HIF, the levels of which are stabilized by hypoxia (see Ref. 1 for review). The oxygen-sensing mechanism is also sensitive to iron chelation. It has been shown that the iron chelator deferoxamine mesylate is capable of inducing hypoxia-regulated gene expression (2, 3). A key mechanism for oxygen sensing has recently been shown by two groups (4, 5) to be a proline hydroxylase, which is an iron containing tetramer. Iron chelation inhibits activity of this enzyme (4, 5) and prevents the post translational modification of HIF that targets it for degradation by the von Hippel-Lindau complex.

Exochelins are iron chelators secreted by pathogenic strains of Mycobacterium tuberculosis. They are both lipid and water soluble, rapidly enter cells, have a high binding affinity for ferric iron, and prevent iron-mediated redox reactions (6–9). The exochelin used in our study was the synthetic form of DFE 772 SM (Keystone Biomedical, Inc.). The DFE 772SM was obtained from the Cancer Research United Kingdom cell services and passed at confluence once a week in E4 media supplemented with 10% FCS and glutamine. Chinese hamster ovary (C4.5) cells and a mutant clone Ka13, which does not express HIF, were developed by the Erythropoietin Group, Institute of Molecular Medicine, John Radcliffe Hospital (Oxford, United Kingdom; Ref. 13). HuDMECs were obtained from the Cancer Research United Kingdom cell services and passed at confluence in MCD4B13 media (cell services) supplemented with 10% FCS, glutamine, heparin (5 IU), and 30 μg/ml endothelial cell growth supplement (Sigma, Dorset, United Kingdom).

Cell Proliferation Assays. Cell counts on HuDMEC at various time points were performed in a Coulter counter (Beckman Coulter, High Wycombe, United Kingdom) after trypsinization and resuspension in buffered solution. Cells were grown in 6-well plates, media pulsed with VEGF (8 ng/ml) with or without DFE 10 μM on days 0, 2, and 4.

Normoxic/Hypoxic Incubation. Cells were exposed to normoxic conditions (Forma Scientific CO2 water jacketed incubator) at 37°C, 5% CO2, and 21% oxygen. Hypoxic conditions were maintained for 16 h at 37°C, 95% humidity, 5% CO2, and 0.1% oxygen in nitrogen-flushed hypoxic chambers (Heto Cellhouse 170 HI).

Exochelin Drug. DFE 772SM and iron-loaded exochelin (ferri-exochelin 772SM) were a gift from Keystone Biomedical, Inc. The DFE 772SM was dissolved in 0.09% saline, sonicated in a water bath, and heated to 90°C, giving a stock solution of 0.5 mg/ml contained in an iron-free plastic tube. The stock solution was stored at 4°C. Similar preparations were made of ferri-exochelin. During experiments, iron-free minimal essential media were used during the exposure of cell samples to DFE to maximize the intracellular iron-chelating effects.

Western Blotting for HIF-1α and NIP3 Proteins. Cells were lysed in 8 M urea lysis buffer, and homogenized lysates were standardized for protein levels (Bio-Rad protein assay kit; Bio-Rad Laboratories, Hercules, CA) before separation on 6% SDS polyacrylamide gels and transferred onto Immobilon membranes (Millipore, Bedford, MA) with semidry blotter Imm-2 (W. E. P. Co., Concord, CA). Membranes were blotted with primary antibodies mouse antihuman HIF-1α antibody 1:1000 (Transduction Laboratories, Becton Dickinson United Kingdom, Oxford, United Kingdom) and Anti-NIP3 antibody (a gift from Arnold H. Greenberg, The Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Canada). Horseradish peroxidase-conjugated goat antimouse antibody (Dako, Glostrup, Denmark) was applied at 1:1000 and chemiluminescence detected with enhanced chemiluminescence Western blotting kit (Amersham, Buckinghamshire, United Kingdom). After

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1 L. D. H. has declared a financial interest in the company Keystone Biomedical, Inc., whose product DFE 772 SM was studied in this work.

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3 The abbreviations used are: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; ARNT, aryl hydrocarbon nuclear translocator; HRE, hypoxia response element; HuDMEC, human dermal microvascular endothelial cell; PGK, phosphoglycerate kinase; DFE, desferri-exochelin.
Exochelin Stabilizes HIF in MDA468 Breast Cancer Cells. Sixteen h exposures to either hypoxia 0.1% or DFE 772SM caused stabilization of HIF-1α protein detected by Western blots in MDA468 breast cancer cells (Fig. 1A). Concomitant exposure to both hypoxia and DFE did not reveal any significant additive effects on HIF-1α expression. Exposure to iron-loaded exochelin did not result in stabilization of HIF-1α. Stabilization of HIF-1α by DFE occurred by 1 h, reached maximal levels by 4 h, and did not increase further between 4 and 10 h exposure (Fig. 1B). We found that the DFE stabilized HIF-1α at doses 1 log less than were required for deferoxamine for the same effect (Fig. 1C). Similar results for HIF-2α (data not shown).

Exochelin Induces VEGF in MDA468 Breast Cancer Cells. VEGF levels were measured by ELISA in culture media from the same samples assayed in Fig. 1. Exposure to either hypoxia or DFE increased VEGF levels compared with levels during normoxia alone. There were no differences in VEGF levels among exposures to hypoxia, DFE, or the combination of hypoxia and DFE. Exposure to ferri-exochelin did not increase VEGF levels significantly (Fig. 2A).

Therefore, iron chelation was required for induction of VEGF by DFE. RNase protection assays were performed on MDA468 cells exposed to 10 μM DFE 772SM with normoxia as control. After correction for loading variation, there was a 3–6-fold induction of VEGF transcripts with exposure to hypoxia, DFE, or hypoxia/DFE compared with normoxia alone. There were no differences in VEGF RNA expression among the samples from the three exposures (Fig. 2B). The protection bands appear as a doublet, with the upper band corresponding to the VEGF 121 amino acid isoform and the lower to the VEGF 165 amino acid isoform. These isoforms arise from alternative exon splicing and have different biological activities. The VEGF 121 riboprobe protects a 517 nucleotide sequence in the VEGF 121 transcript but only 439 nucleotides in the VEGF 165 transcript because of interrupted protection from an intervening noncomplementary sequence. Hence, the protected VEGF 121 transcript migrated slower than the bigger isofrom VEGF 165. In C4.5 cells, exposure to DFE resulted in induction of luciferase-HRE PGK (Fig. 2C). In HIF-1α-mutated Ka13 cells, no increase in luciferase expression was noted in cells exposed to DFE. However transfection of HIF-1α back into Ka13 cells restored induction of luciferase-HRE PGK by DFE. Therefore, HIF-1α was required for HRE induction by DFE.

Exochelin Induces Preactopoptic Protein NIP3 in MDA468 Breast Cancer Cells. We performed Western blots for NIP3 after MDA468 cells were treated with various doses of DFE over 16 h, with normoxic and hypoxic controls. Although NIP3 protein is calculated to have a molecular mass of 21 kDa, transiently expressed NIP3 protein has been shown to migrate as both 30 and 60 kDa forms (14). Induction of both 30 and 60 KDa forms of NIP3 by DFE was detected from 0.5 μM concentration (Fig. 3A). To compare the dual effects of exochelin on VEGF and NIP3 induction in MDA468 cells, we compared VEGF levels measured by ELISA, with Western blots for NIP3 expression (Fig. 3B). Both VEGF and NIP3 showed similar induction over normoxic controls from 0.5 μM exochelin, with increasing expression at higher doses (1.0 and 10 μM).

Exochelin Inhibits Endothelial Cell Proliferative Response to VEGF. To assess the effects of DFE on endothelial cell growth in the presence of the angiogenic growth factor VEGF, we measured cell proliferation in HuDMECs. DFE prevented VEGF-induced cell proliferation (Fig. 4).

DISCUSSION

We showed that DFE 772SM can stabilize HIF and that this effect was dose dependent under normoxic conditions. DFE probably acts at least partly through the same pathway as hypoxia in view of the lack of an additive effect when both were present. The addition of iron to a DFE eliminated its ability to induce HIF. Therefore, this action of...
DFE is solely related to iron chelation. DFE stabilized HIF within 1 h. It is likely that the lipid solubility of DFE allowed it to quickly enter cells and bind iron intracellularly. In support of this, we have also shown that DFE required a smaller dose (1 log less) to achieve a similar effect to the lipid-insoluble iron chelator deferoxamine, which does not readily enter cells. Pahl et al. (10) have similarly shown that this DFE was 10 times more effective than deferoxamine in inhibiting vascular smooth muscle cell proliferation. Horwitz et al. (9) reported that the relative protective effects of different forms of DFE in cardiac myocytes exposed to hydrogen peroxide corresponded to their lipid solubility, with the most lipid-soluble forms having the greatest effect. The increased intracellular iron binding ability of DFE is believed to be attributable to its enhanced diffusion across lipophilic cell membranes.

Hypoxia induces a complex program of gene expression involving both proapoptotic and antiapoptotic pathways (15–18). We studied both a pathway known to enhance angiogenesis and tumor growth (VEGF) and a pathway that promotes tumor cell death by apoptosis (NIP3). Both pathways were activated by iron chelation with DFE. NIP3 is a mitochondrial protein that is induced by hypoxia (12, 14), and we now report that it is induced by iron chelation as well. Recently, we showed that hypoxia may regulate a specific necrosis pathway via NIP3 (14). Hypoxia selects for survival of more aggressive cancer cell variants that express mutant p53 (16). Because NIP3 does not require p53 for apoptosis, induction of NIP3 by DFE may be of therapeutic value. Additionally, iron deprivation may have other effects on cancer cell growth, so HIF induction of NIP3 may not be the sole pathway by which DFE kills cancer cells. Pahl et al. (11) demonstrated that iron chelation with DFE 772SM induces cell death by apoptosis in cultured breast cancer cells but not in normal breast epithelial cells. We have also demonstrated iron chelation-dependent inhibition of MDA468 proliferation by DFE through increased cell death independent of HIF, with no effects on cell cycle (data not shown).

We found that by stabilizing HIF with DFE, both VEGF and NIP3 were up-regulated in MDA468 tumor cells at the same levels of DFE (0.5 μM). We demonstrated transcriptional up-regulation of VEGF by DFE, and this effect has also been demonstrated to be HIF dependent. This precludes a therapeutic window at which proapoptotic effects via NIP3 could occur while minimizing VEGF induction. Notwithstanding this, DFE and its derivatives offer promise as new therapeutic agents for the treatment of cancer.

**Fig. 1.** (A) Stable HIF levels in MDA468 cells as determined by luciferase activity. Samples in duplicate; comparison of means (media alone versus DFE treated) using two-tailed independent Student’s t test (*P < 0.05). (B) Western blot for VEGF (30 and 60 kDa) and HIF (120 kDa) protein with similar escalating doses.
ing, the presence of VEGF did not prevent the inhibitory effects of DFE on endothelial cell growth. Therefore, DFE exhibited antiangiogenic effects, although VEGF induction occurs.

The novel iron chelator exochelin, in its DFE form, could potentially target both hypoxic and normoxic areas of the tumor. Its proapoptotic effects via NIP3 may be additionally enhanced by HIF-independent hypoxia-inducible gene expression. However, DFE could be antiangiogenic in vivo despite the induction of VEGF by targeting tumor endothelial cell proliferation. The superior efficacy of DFE over deferoxamine could make it more suitable for use in clinical trials.

REFERENCES


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