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Iron depletion decreases proliferation and induces apoptosis in a human colonic adenocarcinoma cell line, Caco2

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ABSTRACT

Iron is essential for maintaining cellular metabolism of most organisms. Iron chelators such as desferrioxamine have been used clinically in the treatment of iron overload diseases. In the present study, we used human colon adenocarcinoma cells as a proliferating cell model to validate that desferrioxamine inhibits cell proliferation and induces apoptosis. Proteomic analysis revealed that proteins involved in cell proliferation, signal transduction, metabolism and protein synthesis were significantly regulated by the availability of iron, rendering a close correlation between cell apoptosis and the disturbance of mitochondrial, signaling and metabolic pathways. These results provide new insights into the mechanisms of cell proliferation inhibition attributed to iron depletion.

1. Introduction

Iron (Fe) is essential for a variety of important biological processes, including DNA synthesis, respiration, oxygen transport, and energy production [1,2]. Organisms need to take up large amounts of Fe from the environment or the host to help maintain critical cellular metabolism and support growth [3]. Tumor growth is enhanced by Fe, as observed in cell culture, animal and human studies [4]. Fe withdrawal strategies have been investigated for their potential roles in the management of human tumors and Fe overload diseases [5]. Desferrioxamine (DFO), one of the most widely studied Fe chelators to date, has been successfully applied in antineoplastic therapy [6–10]. The efficiency of these Fe chelators in inhibiting malignant cell proliferation arises from their ability to deplete Fe that is preferentially needed by rapidly growing tumor cells for DNA synthesis and metabolism.

It has been reported that Fe depletion by chelators typically results in cell cycle arrest and programmed cell death or apoptosis in various kinds of cell lines [11–14]. At molecular levels, recent studies have demonstrated that Fe depletion can lead to complicated responses affecting not only ribonucleotide reductase, but also a range of molecules responsible for cell cycle control, such as $p21^{CIP1/WAF1}$ [15]. Fe chelator-treated cells were arrested in different phases of the cell cycle, depending on the cell type, the type and treatment concentration of chelators and the drug-exposure time. Moreover, Fe chelator treatment resulted in down-regulated expression of anti-apoptotic molecule Bcl-2 and up-regulated expression of pro-apoptotic molecules including Bax, and led to apoptotic cell death [14,16]. Nevertheless, the detailed molecular mechanism underlying the cell growth inhibition by Fe chelators is not yet fully understood in the proteomic scale.

Colorectal cancer (colon cancer) is one of the most prevalent cancers in the West [17]. Many colorectal cancers are thought to arise from adenomatous polyps in the colon. Derived from a human colon carcinoma, Caco2 cells were used as a model cell culture system in this study. The anti-proliferative effects of Fe chelator DFO on Caco2 cells were analyzed by using proteomic and biochemical methods. Proteomics has been proven to be an effective approach for the study of complex biological systems [18], and has been extensively employed to evaluate the proteomic response of cells to drug treatment [19–21]. By comparing protein profiles between untreated and DFO-treated Caco2 cells in the presence or absence of ferric ammonium citrate (FAC), consistently and significantly regulated proteins were identified by peptide mass finger-printing (PMF) and further confirmed by Western blotting analysis. The current results provide new insights into the potential mechanism of the effects of Fe depletion on cell proliferation and apoptosis in human carcinoma cells.

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2. Materials and methods

2.1. Cell culture and treatment

Caco2, a human colon adenocarcinoma cell line, was generously provided by Prof. Marie Chia-Mi Lin (Department of Chemistry, The University of Hong Kong), and was cultured in Dulbecco's modified Eagle medium (Gibco-BRL, Paisley, Scotland), supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (100 U/mL). Cells were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2.

When cultures reached 60% confluence, they were harvested and plated for either subsequent passage or drug treatment. Cultures were treated with DFO at a series of concentrations (50–1000 μM) for 3 days. In an additional assay, FAC was added simultaneously with DFO in one to one molar ratio to maintain iron concentration in the media to verify that the inhibitory effects of DFO are due to its iron chelator capability. Cell viability was determined by Naphthol blue black (NBB) staining assay, according to the method previously reported [22].

2.2. Fluorescence microscopy

To identify morphological changes during apoptotic process, nuclear staining was carried out with 4,6-diaminino-2-phenylindole (DAPI) (Sigma, St. Louis, MO). Briefly, cells were fixed with 4% formaldehyde, washed with methanol, stained with 0.5 μg/ml DAPI in methanol, and then observed under a fluorescence microscope (Olympus IX71 Chinetek Scientific Microscope). The percentage of apoptotic cells was calculated by counting the number of apoptotic cells relative to total number of cells in a viewing field. Cell counts were determined over six viewing fields and averaged.

2.3. DNA 5-bromo-2′-deoxyuridine (BrdU) incorporation

BrdU (Calbiochem, Sweden) incorporation during DNA replication was determined by an indirect immunocytochemistry method in accordance with manufacturer's recommendations. Cells were incubated with BrdU which was diluted in culture medium (1:2000), at 37 °C for 4 h. The cells were fixed for 30 min in a solution of 90% ethanol and 5% acetic acid, and then washed three times with phosphate-buffered saline (PBS). BrdU was recognized with a mouse anti-BrdU monoclonal antibody, which was further detected using a secondary anti-mouse IgG2A immunoglobulin coupled to horseradish peroxidase. Positive cells were then revealed with tetra-methylbenzidine.

2.4. Cell cycle analysis by flow cytometry

Cells were treated with 200 μM DFO for various periods of time. After treatment, cells were harvested, resuspended in PBS and finally fixed in cold ethanol at 4 °C. Cells (5×10^5) were stained with propidium iodide (PI) for 30 min in the dark and immediately analyzed on a FACStar Plus flow cytometer. Data analysis was carried out with ModFit LT software.

2.5. Caspase activity assay

Caspase-3 and caspase-9 activities were determined by fluorometric caspase activity assay kits (Oncogene, San Diego, CA), following the protocols recommended by the supplier.

2.6. Subcellular fractionation

Whole cell lysate was obtained by resuspending cells in a lysis buffer (8 M Urea, 4% CHAPS, 2% IPG Buffer and 0.2 mg/ml PMSF). To obtain nuclear proteins, harvested cells were incubated on ice for 10 min in an extraction buffer (10 mM Tris–HCl (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF and 0.1% protease inhibitor cocktail (Oncogene)), and then Triton X-100 was added to a final concentration of 0.1% (v/v) [23]. The mixture was further incubated for another 1 min. The crude lysates were centrifuged at 700 *g* for 10 min to remove nuclei. The supernatant was retained and centrifuged at 10,000 *g* for 30 min at 4 °C to obtain the cytosolic fraction, and the resulting pellet was saved as mitochondria-containing heavy membrane fraction. Consistent with the results in our previous study [23], the effect of the low concentration Triton X-100 on the integrity of the mitochondria was undetectable; intact mitochondria were verified by Western blotting with an antibody against the specific mitochondrial marker COX-IV.

2.7. Mitochondrial transmembrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated by a lipophilic cationic probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, USA) as described in a previous report [24]. During the last 30 min of cell culture with or without the treatment of 200 μM DFO, 5 μM JC-1 was added to the media. $\Delta\Psi_m$ depletion was observed under a fluorescence microscope. A green filter was used for green-fluorescent monomer at depolarized membrane potentials, and a red filter for orange-fluorescent J-aggregate at hyperpolarized membrane potentials.

2.8. Two-dimensional electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was carried out using an IPGphor II isoelectric focusing (IEF) unit and a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare) according to a previously reported method [18]. Briefly, 100 μg of proteins, extracted from Caco2 cells untreated or treated with 200 μM DFO in the presence or absence of FAC for 72 h, was diluted in a rehydration solution (8 M urea, 4% CHAPS, 1 mM PMSF, 20 mM DTT and 0.5% IPG buffer). IEF was carried out with precast 13-cm Immobiline DryStrips (immobilized pH gradient strips; GE Healthcare) to generate a nonlinear pH gradient from 3 to 10. After IEF, the strips were transferred to 1.5-mm thick 12.5% polyacrylamide slab gels and electrophoresed. Triplicate experiments were performed to ensure reproducibility. All gels were visualized by silver-staining [18].

2.9. Image analysis and MS–PMF

Silver-stained gels were scanned by using an ImageScanner (GE Healthcare) operated by the LabScan 3.00 software. Intensity calibration was performed with an intensity step wedge prior to gel image capture. Image analysis was performed with Image master 2D Elite software 4.01 (GE Healthcare) [18]. Image spots were initially detected, matched, and then manually edited. Each spot intensity volume was processed by background subtraction and total spot volume normalization, and the resulting spot volume percentage was used for comparison. Only those significantly and consistently altered protein spots in at least three independent experiments (over 2-fold up- or down-regulation) were selected for analysis by MS. In-gel trypsin digestion, MS analyses and database searching basically followed the method previously described [18]. Duplicate or triplicate runs were made to ensure the consistency of the analyses.

2.10. Western blotting

Immunoblotting analysis was performed using primary antibodies against Rho-GDI, cyclophilin A (CypA), and β-tubulin (Up-