Iron deprivation-induced apoptosis in HL-60 cells

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Abstract
Iron deprivation of HL-60 cells with deferoxamine B mesylate (DFO) induced apoptosis. DNA fragmentation became apparent with 10^-6 M DFO after 48 h treatment. The apoptosis peak according to the DNA histogram on flow cytometry and typical nuclear collapse and were observed microscopically after 48 h treatment with 10^-4 M DFO. Cells treated with 10^-4 M DFO for as little as 24 h were shown to be committed to apoptosis, as chromatin condensation progressed gradually thereafter.

Key words: Deferoxamine B mesylate; Apoptosis; DNA fragmentation

1. Introduction

Iron chelation has been shown to cause S-phase inhibition of cell proliferation [1]. The iron chelating reagent, deferoxamine B mesylate (DFO) is reported to enter cells and remove iron from the intracellular pool [2]. Since iron is essential for the activity of ribonucleotide reductase, which is responsible for the synthesis of deoxyribonucleotides, cell growth was inhibited after DFO treatment [1,3]. Recently we reported that intracellular iron deprivation caused by DFO treatment increased the expression of c-myc and c-fos protooncogenes in coordination with the growth arrest of cells in the S phase [4-6]. The expression rates of c-fos and c-myc were shown to be enhanced after growth stimulation by serum, growth factor, 12-O-tetradecanoyl phorbol-13-acetate, or after induction of cell apoptosis [10]. Also, overexpression of c-myc was shown to induce apoptosis [11]. In the present report we show apoptosis may be induced by DFO treatment.

2. Materials and Methods

2.1. Cells
Human myeloblastic leukemia cell line, HL-60 (supplied by JCRB), were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin. Experiments were performed with exponentially growing cells at 3.6 x 10^6 cells/ml.

2.2. Reagents
Deferoxamine B mesylate (DFO, M, 656.8 Ciba-Geigy, Basel, Switzerland) was diluted into the culture medium immediately before use.

2.3. DNA extraction and gel electrophoresis
Cells were lysed in guanidine-isothiocyanate and DNA was extracted by benzyl chloride using the SepaGene kit (Sanko, Tokyo). After any remaining RNA was digested with 1 μg/ml RNase A, 50 μg of each DNA sample was loaded on to a 1.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

2.4. Flow cytometry
The cells were fixed in 50% ethanol, treated with 0.1 mg/ml RNase A, and stained with 100 μg/ml propidium iodide. Cell cycle profiles and distributions were determined by flow cytometry, FACScan (Becton Dickinson, CA).

2.5. Microscopic analysis
Cells were cytocentrifuged and stained by the Papanicolau method.

3. Results

3.1. DNA fragmentation
HL-60 cells were treated with 10^-6, 10^-5 and 10^-4 M DFO for 24, 48 or 72 h. Fig. 1 shows the electrophoresis pattern of cellular DNA after treatment with DFO. DNA fragmentation became apparent within 48 h of 10^-6 M DFO treatment. This pattern was more pronounced following treatment at 10^-5 or 10^-4 M and with longer incubation times (Fig. 1). To evaluate that the effect was due to the iron deprivation with DFO, we preincubated DFO with equal amount of FeCl_3. The iron loaded DFO did not cause the DNA fragmentation (data not shown).

3.2. PI staining pattern
Fig. 2 demonstrates the DNA histograms of DFO-treated cells. With 3 x 10^-5 M DFO treatment for 24 h, the accumulation of cells in the early S phase was observed (Fig. 2). With 48 h, 10^-4 M DFO treatment, the typical apoptosis pattern of cells containing a small amount of DNA, was demonstrated with a pronounced loss of cells in the S and G2M phase (Fig. 2).

3.3. Microscopic analysis
To examine the chromatin structure in detail, we employed the Papanicolau staining method. The abnormal chromatin clumps and nuclear membrane wrinkling had

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Fig. 1. DNA fragmentation analysis after deferoxamine treatment. Fifty μg of cellular DNA was electrophoresed on a 1.8% agarose gel. Similar findings were obtained in three separate experiments.

appeared after 24 h treatment with $10^{-5}$ M and $10^{-4}$ M DFO (Fig. 3). Nuclear collapse appeared after 48 h treatment with $10^{-5}$ M DFO (Fig. 3).

3.4. Determination of the period of DFO treatment for commitment to apoptosis

We tried to determine the period of treatment required for DFO-induced apoptosis. After cells were treated for 6, 12 or 24 h with $10^{-4}$ M DFO, the DFO-containing medium was replaced with fresh medium. Cells were cultured further for a total of 48 h, and their DNA histogram were studied. As shown in Fig. 4a, loss of G2M phase cells apparent after a 6 h treatment; however, no apoptosis peak was observed after 24 h treatment. The apoptosis peak became apparent when cells treated with $10^{-4}$ M DFO for 24 h were cultured further with fresh medium for 24 h (Fig. 4b). Thus the cells were committed to apoptosis after they had been exposed to DFO for at least 24 h.

4. Discussion

We have demonstrated that the DFO treatment caused the inhibition of cell growth and induced apoptosis manifested as DNA fragmentation on electrophoresis and on DNA histogram and microscopic analyses. We observed recently that c-myc and c-fos expression rates increase after treatment with DFO, and felt this represented the process of apoptosis [4-6]. Increase of c-myc and c-fos expression preceding apoptosis was reported by several investigators and our results concur [7-10].

Apoptosis caused by iron deprivation was moderate compared to that caused by various antitumor
agents [12]. We presume that the response to DFO was more moderate due to the fact DFO treatment did not damage DNA directly. Iron chelation deactivates ribonucleotide reductase, perturbs the nucleotide pool, and thereby inhibits DNA synthesis.

DNA fragmentation becomes evident prior to any change in the DNA frequency histogram or apparent change in chromatin structure. This result confirmed that the DNA cleavage was an early event leading to apoptosis in this system.

Iron deprivation caused by $10^{-4} \text{ M}$ DFO treatment for 24 h may commit cells to apoptosis. Thereafter, typical apoptosis bodies formed gradually even in a medium without DFO. Due to the pace of this apoptosis being moderate, we would be able to analyze the mechanism of chromatin condensation process.

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