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The Mechanism of Antiproliferative Effect of Desferrioxamine on Human Hepatoma Cell Lines

Doe Young Kim[1], Won Ho Kim, Jin Kyung Kang
In Suh Park and Oh Hun Kwon[2]

We investigated the effect of desferrioxamine (DFO), an iron chelator, on the DNA synthesis and the cell cycle of cultured hepatoma cells. Using Hep 3B cells as the hepatoma cell lines, DNA synthesis was measured by [^{3}H] thymidine incorporation, and the cell cycle analysis was performed by flow cytometry including bivariate DNA/BrdU analysis. [^{3}H] thymidine uptake was decreased by DFO in a dose dependent manner. The proportion of S phase cells increased and that of G0/G1 phase cells decreased after the addition of DFO in the culture media in a dose dependent manner up to 20 μg/ml of DFO. The S phase duration of the exponentially proliferating Hep 3B cells was 9.9 hours when cultured without DFO, but it was markedly prolonged (54.1 hours) after the addition of 20 μg/ml of DFO. After removal of DFO from the culture media following 24 hours of incubation with 20μg/ml of DFO, a sequential increase from early through mid and late-S to G2/M phase was observed. In conclusion, the antiproliferative effect of DFO on cultured human hepatoma cell lines was caused by the inhibition of DNA synthesis which was related to a block in the early-mid S interface or mid S phase of the cell cycle.

Key Words: Hepatoma cell lines, desferrioxamine, cell cycle

Iron is essential for the growth of all living cells including tumor cells. However, iatrogenic iron overload has been associated with neoplasia; parenteral administration of iron dextran induced sarcomas at the sites of injection in rodents, rabbits, and humans (Goldberger *et al.* 1960). Based on these obervations, it was postulated that the depletion of iron might exert an antitumor effect (Hann *et al.* 1988).

Desferrioxamine (DFO), an iron chelator, has been demonstrated to inhibit the proliferation of normal human hematopoietic cells (Lederman *et al.* 1984), a variety of malignant cell lines (Foa *et al.* 1986; Blatt and Stitely 1987), and human bone marrow neuroblastoma cells (Becton and Bryles 1988). Additionally, DFO has been shown to have antitumor activity against cultured cell lines of human hepatocellular carcinoma which is one of the most common causes of death due to cancer in Korea (Hann *et al.* 1990; Tabor and Kim 1991; Song 1992).

DFO has been reported to decrease ribonucleotide reductase activity resulting in decreased DNA synthesis in human lymphocytes (Lederman *et al.* 1984). The cell cycle analysis of mitogen-stimulated lymphocytes revealed that the cells were blocked early in the S phase of the cell proliferation cycle (Lederman *et al.* 1984). However, the effect of DFO on the cell cycle varies according to the kinds and status of the cells (Hedley *et al.* 1985; Reddel *et al.* 1985). Though an understanding of the mechanisms involved is nec-

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Department of Internal Medicine[1], Ewha Womans University Medical College, Seoul, Korea
Department of Internal Medicine, Clinical Pathology[2], Yonsei University Medical College, Seoul, Korea
Address reprint requests to Dr. DY Kim, Department of Internal Medicine, Ewha Womans University Medical College, Seoul, Korea

essary for DFO to be used as a modulator of tumor growth or as an adjunctive mode of treatment with cell cycle-specific chemotherapeutic agents, the effect of DFO on DNA synthesis and the cell cycle of human hepatoma cells has yet to be clarified.

This study was conducted to investigate "whether DFO inhibits the DNA synthesis of cultured hepatoma cells" and "what effect does DFO have on the cell cycle of these cells". Using Hep 3B cells as the hepatoma cell lines, DNA synthesis was measured by [^{3}H] thymidine incorporation, and bivariate DNA flow cytometry, after staining with propidium iodide (PI) and anti-bromodeoxyuridine (BrdU) following BrdU labeling, was performed to analyze the cell cycle.

MATERIALS AND METHOD

Cell culture

Hep 3B cells were purchased from ATCC (American Type Culture Collection; Rockville, MD, U.S.A.), and cultured at 5% CO_2, 37℃ in Eagle's MEM (minimal essential media; JR Scientific & Hazleton Biologic Inc., Lenexa, KS, U.S.A.) containing 10% (v/v) fetal calf serum (Commonwealth Serum Laboratories Ltd., Parkville, Australia), 100 IU/ml of penicillin, 100 μg/ml of streptomycin in a 25 cm^2 plastic flask (Costar, Cambridge, MA, U.S.A.). The same lot number of fetal calf serum (lot no. 0971302) was used during the whole experiment to maintain the same concentration of iron in the medium.

DFO treatment

After dispensing Hep 3B (2.4×10^4 cells/well) cells into 24-well plates, 0, 2, 6, 20, 60 or 120 μg/ml of DFO was added and incubated for 2, 24, 48, 72 or 96 hours.

Antiproliferative effect and [^{3}H] thymidine incorporation

The cells were counted in a haemocytometer and viability was assessed by exclusion of 0.2% Trypan blue. [^{3}H] thymidine incorporation was done to measure DNA synthesis. Following a 2- to 72- hour treatment with various concentrations of DFO, 100 μl of cell suspensions (1×10^4 viable cells/ml) were plated in 96-well plates. Cells were then pulsed with 1 μCi of [^{3}H] thymidine (20 Ci/mmol; 740 GBq/mmol, NEN, Wilmington, DE, U.S.A.) for 1 hour. Cells were harvested with a cell harvestor (Skatron Inc., Stering, VA, U.S.A), collected on filter paper and dried. The uptake of radioisotopes was measured with liquid scintillation, and the results were expressed as the mean of three counts per minute (cpm) among the pentaplicate values, ommitting the upper and the lower most value. The percent inhibition of [^{3}H] thymidine uptake was calculated by

$$\% \text{ Inhibition} = \frac{\text{Control cpm} - \text{DFO treated cpm}}{\text{Control cpm}} \times 100$$

Flow cytometric DNA analysis

After incubation for 2 to 72 hours with various concentrations of DFO, the cells were centrifuged at 500×g for 5 minutes at room temperature; the supernant was then discarded and the pellets were washed twice with 5 ml of phosphate buffered saline (PBS) or citrate buffer (250 mM sucrose, 40 mM trisodium citrate; pH 7.6). The cells were filtered through a 53 μM nylon mesh filter (Spectrum, Houston, TX, U.S.A.) to prevent cell clumping. Solution (A) (trypsin 15 mg, stock solution to 500 ml), solution (B) (trypsin inhibitor 250 mg, ribonuclease A 50 mg, stock solution to 500 ml), and solution (C) (PI 208 mg, spermine tetrahydrochloride 580 mg, stock solution to 500 ml) were sequentially added and mixed. Fluorescence was measured after laser excitation at 488 nm with a FACScan flow cytometer (Becton Dickinson Immunocytometry System, Mountain View, CA, U.S.A.). More than 10^4 cellular events were measured, and the obtained DNA histogram was analyzed with the RFIT (Rectangle Fit; S phase less than 20%) or SFIT (S phase Fit; S phase more than 20%) program. The composition of the used stock solution (pH 7.6) was as follows;

3.4 mM trisodium citrate
0.1% (v/v) Nonidet P-40
1.5 mM spermine tetrahydrochloride
0.5 mM tris (hydroxymethyl)-aminomethane

Bivariate flow cytometry using anti- BrdU antibody

The incubation time, 24 hours, and the concentration of DFO, 20 μg/ml, which had had a major impact on the cell proliferation and the cell cycle in the preliminary experiment, was chosen and used for the following experiment.

Pulse labeling with BrdU

Hep 3B cells (1×10^6 cells) were incubated for 24 hours with or without 20 μg/ml of DFO, and then pulse labeled with 10 μM of BrdU (Sigma Chemical Co., St. Louis, MO, U. S.A.) at the end of incubation for 20 min. At the end of incubation, cells were washed in PBS and resuspended in fresh medium. At the specific recovery times (0, 2, 4, 6 and 8 hours), cells were fixed in 70% cold ethanol and stored at 4℃.

PI-antiBrdU staining technique

The cells were stained by the PI and anti-BrdU direct immunofluorescence method, developed by Dolbeare *et al.* (1985). The ethanol-fixed cell suspensions were centrifuged and incubated with 2 N HCl/Triton X-100 for 30 min to obtain partially denatured DNA. After washing with 0.1 M $Na_2B_4O_7$ (pH 8.5) to neutralize acid, the cell pellets were resuspended with 50 μl of Tween 20 (Merck) 0.5 % in PBS. After that, 50 μl of bovine serum albumin 0.5% in PBS and 20 μl of fluorescein isothiocyanate (FITC) conjugated anti-BrdU antibody (Becton Dickinson Immunocytochemistry Sytstem) were added and incubated for 30 min at room temperature. At the end of antibody exposure, the cells were centrifuged, resuspended in 5 μg/ml of PI in PBS, filtered through a nylon mesh and analyzed on a FACScan with standard (FITC/PI) filter sets.

Analysis of the cell cycle phase distribution

Five phase compartments in the cell cycle were defined according to DNA content and BrdU incorporation as suggested by Fujikane *et al.* (1989). DNA content was divided into three compartments, 2C, 3C, and 4C. The 2C or 4C cells were defined as the cells whose fluoroscence intensity varied between -3 standard deviation (SD) and +2 SD around 2C or 4C peak. The 3C cells were those lying between the 2C and 4C. The SD was calculated using the coefficient of variation (CV) of G0/G1 peak and peak channel values (2C or 4C) in a DNA histogram. The five cell cycle phase compartments were defined as follows. The early-S phase cells had 2C, the mid-S phase cells had 3C, and the late-S phase cells had 4C DNA content in the BrdU-incorporating cells. The G0/G1-phase cells had 2C, and G2/M-phase cells had 4C DNA content in the BrdU-nonincorporating cells. Each cell cycle phase distribution was analyzed with the LYSYS II program on a FACScan.

Estimation of relative movement (RM) and S phase duration (Ts)

The relative movement (RM) of the cells was calculated at different times after pulse labeling according to the method of Begg *et al.* (1985). The RM was defined as the mean DNA content of the S-phase (labeled) cells less the mean DNA content of the G0/G1 (unlabeled) cells, divided by the difference between the mean DNA content of G2/M and G0/G1 (unlabeled) cells. S phase duration (Ts) was extrapolated at RM=1.

Reversibility test

The action of DFO was stopped by changing the culture media to DFO free fresh media, following a 24 hour incubation with or without 20 μg/ml of DFO. At 0, 2, 6, 15 and 24 hours after removal of DFO, 10 μM of BrdU was added and incubated for 20 min for bivariate analysis.

RESULT

Antiproliferative effect of DFO

The cell growth rates, expressed as an increase in the number of viable (trypan blue excluding) cells in cultures incubated with various concentrations of DFO are shown in Fig. 1. Hep 3B cells incubated without DFO showed an exponential growth, while the proliferation of the cells was slightly inhibited by 2 μg/ml and markedly inhibited by 6 μg/ml

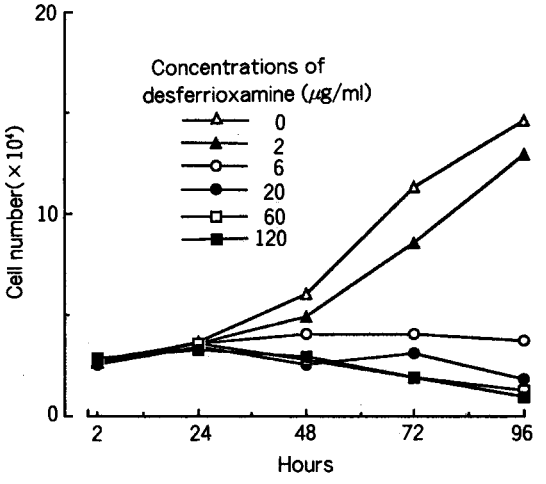

Fig. 1. Growth curves of Hep 3B cells incubated with various concentrations of desferrioxamine. The number of viable cells was measured by the trypan blue dye exclusion method. Each data depicts the mean number of 3 different experiments.

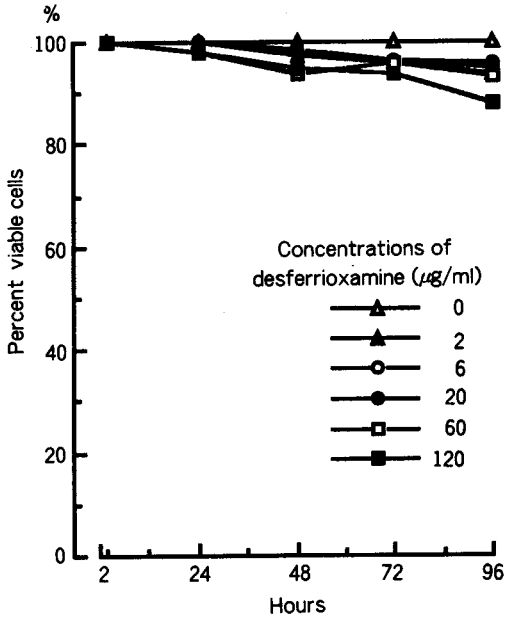

Fig. 2. Percent viability of Hep 3B cells incubated with various concentrations of desferrioxamine. The percent viability was calculated by the percent of viable (trypan blue dye excluding) cells among the total cells. Each data depicts the mean number of 3 different experiments.

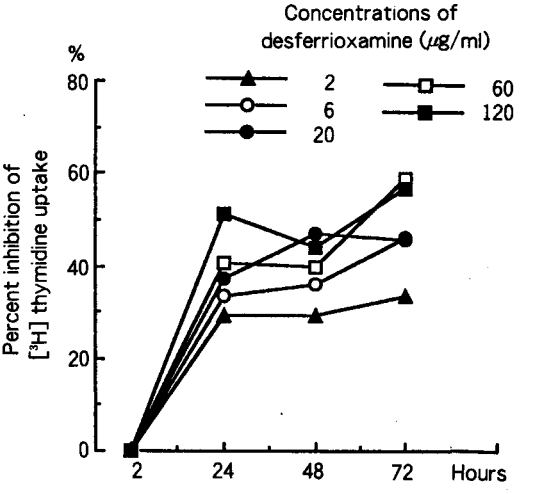

Fig. 3. Percent inhibition of [³H] thymidine uptake of Hep 3B cells incubated with various concentrations of desferrioxamine. The percent inhibition of [³H] thymidine uptake was calculated by the difference between the cpm of control and desferrioxamine treated cells divided by that of control cells at the same incubation time. Each data depicts the mean number of 4 different experiments, each consisting of 5 wells.

of DFO (Fig. 1). Although the cell growth was markedly inhibited, a substantial portion of cells remained viable; the percent viability of Hep 3B cells was above 90% after up to 96 hours of incubation with 60 μg/ml of DFO, and 88.0% after 96 hours of culture with 120 μg/ml of DFO (Fig. 2).

Effect of DFO on [³H] thymidine incorporation

To evaluate the effect of DFO on DNA synthesis, [³H] thymidine incorporation was performed in Hep 3B cells. As shown in Fig. 3, the percent inhibition of [³H] thymidine uptake increased in a dose dependent manner. But according to the incubation time, the plateau was shown after 24 hours of incubation (Fig. 3).

Effect of DFO on the cell cycle

To evaluate the effect of DFO on the cell cycle of Hep 3B cells, each cell cycle phase

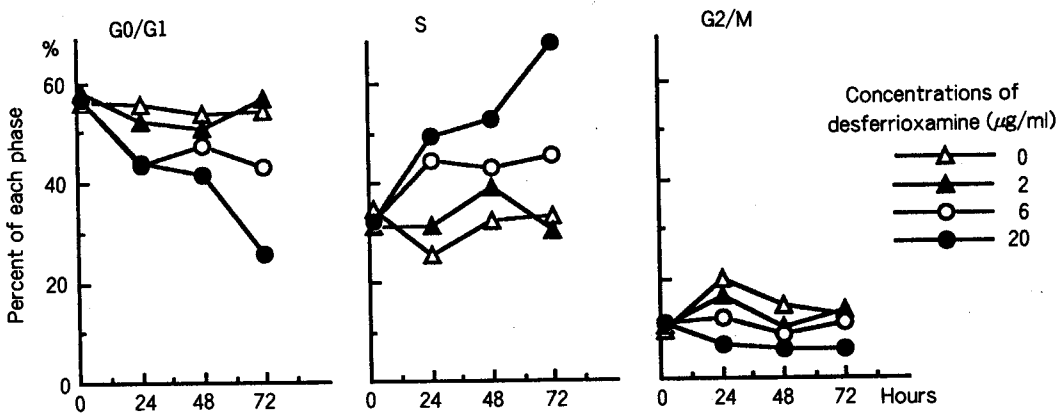

Fig. 4. *Time course of each phase of the cell cycle in Hep 3B cells incubated with various concentrations of desferrioxamine.*

Each phase of the cell cycle was measured by DNA flow cytometry using propidium iodide. Each data depicts the mean percent of 4 different experiments.

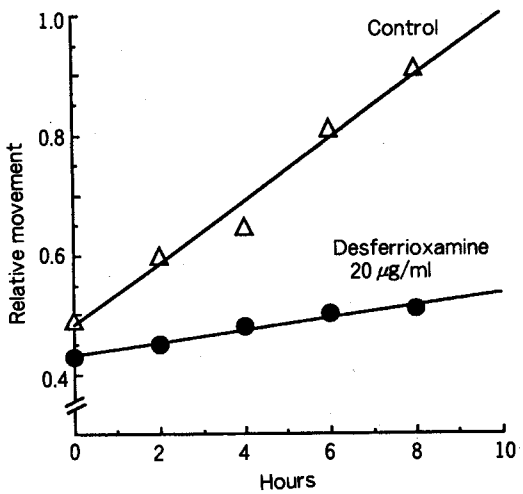

Fig. 5. *Relative movement of Hep 3B cells labeled with BrdU as a function of time.*

Hep 3B cells incubated with or without 20 μg/ml of desferrioxamine for 24 hours were harvested at 0, 2, 4, 6 and 8 hours after labeling with 10 μM of BrdU.

was measured with flow cytometry using PI. Hep 3B cells were diploid. The proportion of G0/G1 phase cells decreased and that of S phase cells increased after the addition of DFO in the culture media in a dose dependent manner up to 20 μg/ml of DFO (Fig. 4).

Effect of DFO on RM and Ts

To evaluate the effect of DFO on RM and Ts, the bivariate flow cytometry was done in Hep 3B cells at 2, 4, 6 and 8 hours after pulse labeling with BrdU, following a 24 hour incubation with or without 20 μg/ml of DFO. After pulse labeling, BrdU labeled control cells (cells incubated without DFO) moved from early toward mid and late-S phase with time progress and the newly divided early-S phase cells appeared at 8 hours. But in DFO treated cells, a continuous accumulation of BrdU labeled cells in the early-S phase was observed, and the cells moved little toward mid-S and late-S with time progress. As shown in Fig. 5, the RM of the control cells increased linearly with time ($y = 0.48629 + 0.05187x$, $r^2=0.983$), and Ts was 9.9 hours, while the RM of DFO treated cells increased little with time ($y=0.432+0.0105x$, $r^2=0.976$), and Ts was markedly prolonged (54.1 hours).

Reversibility of the cell cycle change by DFO

To evaluate the reversibility of the effect of DFO on the cell cycle of Hep 3B cells, the bivariate flow cytometry was performed at recovery times, 0, 2, 6, 15 and 24 hours after

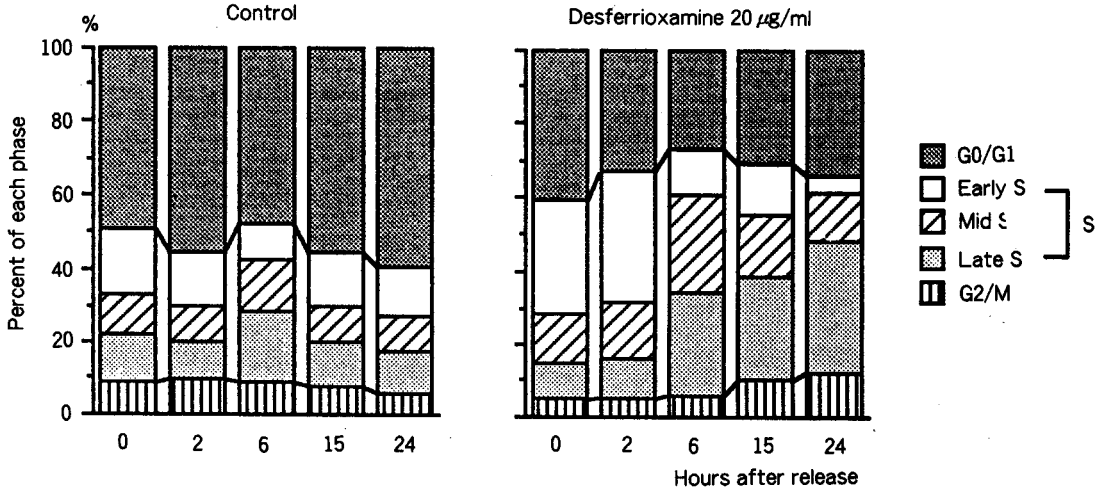

Fig. 6. *Changes in each phase of the cell cycle in Hep 3B cells after 24 hours of incubation with or without 20 μg/ml of desferrioxamine.*

Each phase of the cell cycle was measured by bivariate flow cytometry at 0, 2, 6, 15, and 24 hours after removal of desferrioxamine from the media, following 24 hours of incubation with or without 20 μg/ml of desferrioxamine. Each data depicts the mean percent of 3 different experiments.

removal of DFO from the cultue media. In DFO treated cells, the increase of the S phase and decrease of the G0/G1 phase was still seen at 24 hours after the medium change. However, when the S phase was more specifically divided into early, mid, and late-S phase, a sequential increase from early through mid and late-S to G2/M phase was seen; at 0 and 2 hours, an increase of the early-S phase, at 6 hours, that of the mid-S phase, and at 15 and 24 hours, that of the late-S phase were observed respectively, while control cells showed no specific changes (Fig. 6).

DISCUSSION

Desferrioxamine (DFO), an iron chelator, is a hydroxylamine produced by *Streptomyces pilosus* that enables bacteria to effectively scavenge iron from the environment (Kerberle 1964). DFO has been reported to have antitumor activity against leukemia and neuroblastoma cell lines and malignant cells from patients with acute lymphoid leukemia, acute myeloid leukemia, and neuroblastoma (Bomford *et al.* 1986; Blatt and Stitely 1987; Becton and Bryles 1988). Recently Hann et al. (1990) observed that when human liver cancer cells were incubated in vitro with DFO, DFO exerted cytotoxic effects against these cells. There were several similar reports about the antiproliferative effect of DFO in human hepatocellular carcinoma cell lines (PLC/PRF/5, Hep 3B and Hep G2) in vitro (Tabor and Kim 1991; Song 1992) and in vivo (Hann *et al.* 1992).

For DFO to be used for chemotherapeutic agents, it should have no or little effect against normal cells. Interestingly, the antitumor activity of DFO was seen only in liver cancer cells and not in normal human diploid cells (WI-38 cells) (Hann *et al.* 1990).

DFO has been shown to inhibit DNA synthesis in a human neuroblastoma cell line (CHP-126) (Blatt *et al.* 1988), four human leukemia (HL-60, HEL, HPB-ALL, MOLT 3) or lymphoma (U-937) cell lines (Ganeshaguru *et al.* 1980; Becton and Roberts 1989), a human cervical carcinoma cell line (HeLa) (Robbins and Pederson 1970), PHA-stimulated normal human lymphocytes (Hoffbrand *et al.* 1976;

Ganeshaguru *et al.* 1980), and human thymocytes (Lederman et al. 1984). DFO does not have much direct effect on the synthesis of RNA or protein (Robbins and Pederson 1970; Lederman *et al.* 1984; Blatt *et al.* 1988; Becton and Roberts 1989). However, Bomford *et al.* (1986) found that at the low concentrations, DFO produced little inhibition of DNA synthesis of human leukemic cells (K562) while it apparently and selectively inhibited cell division. In this study, [^{3}H] thymidine incorporation was performed for the measurement of the DNA synthesis in hepatoma cell lines. The precision of the [^{3}H] thymidine uptake method as a measure of DNA synthesis has been questioned by Maurer (1981) and Buchi *et al.* (1991) who cite potential problems associated with variations in precursor pool sizes and changes in thymidine metabolism. Although the uptake of [^{3}H] thymidine may not be precisely synonymous with DNA synthesis, the consistency and reproducibility of the results suggest that the method is an accurate correlation in this study. It revealed that [^{3}H] thymidine uptake was decreased by DFO and that this inhibition of DNA synthesis was dose dependent. Whether this inhibition of DNA synthesis occurs as a result of iron depletion, enzyme inhibition, or some other mechanism, is not known. Ribonucleotide reductase, found in every prokaryotic or eukaryotic cell, provides the balanced supply of four deoxyribonucleotides, and iron and oxygen are needed for it's activity (Thelander and Reichard 1979). DFO has been shown to inhibit iron dependent ribonucleotide reductase in PHA-stimulated human lymphocytes, decreasing the intracellular pool of deoxyribonucleotide triphosphates (primarily dATP) (Hoffbrand *et al.* 1976), and to reduce the intracellular pools of dATP and dGTP in thymocytes (Lederman *et al.* 1984). This might be a possible mechanism for the inhibition of DNA synthesis by DFO in hepatoma cell lines, but it's demonstration in human hepatoma cells require further studies.

DFO has been known to be a reversible blocker in the early S phase of PHA-stimulated human lymphocytes (Lederman *et al.* 1984). The percentage of S phase cells were increased in CCRF-CEM T cell leukemia (Hedley *et al.* 1985), T-47D breast cancer cells (Reddel *et al.* 1985), K562 and HL-60 leukemic cell lines (Bomford *et al.* 1986; Tomoyasu *et al.* 1987) by DFO. However, the effect of DFO on the cell cycle was determined by the kind and status of the tumor cell; CCRF-CEM cells were killed in either the S phase or at the G1-S interface by DFO, while B16 mouse melanoma cells were blocked at different phases (G1 phase) of the cell cycle (Hedley *et al.* 1985). Likewise, untransformed normal rat kidney cells were reversibly blocked in G1 by the iron chelator, while the same agent has been shown to block virus-transformed cells in S or G2 (Fernandez-Pol *et al.* 1977). In hepatoma cell lines of this study, S phase cells were increased and G0/G1 phase cells were decreased in a dose dependent manner by DFO. The plateau was seen after a 24 hour incubation with less than 20 μg/ml of DFO, but the increase of the S phase was time-dependent with 20 μg/ml of DFO. The change of the cell cycle phase by DFO reached a plateau at day 2 - 3 in T-47D breast cancer cell lines(Reddel *et al.* 1985), and at 24 hours in neuroblastoma cell lines(Blatt *et al.* 1988). In T-47D breast cancer cells, L1210 and K562 leukemic cells, low concentrations of DFO caused the block of the G2/M phase and increased the proportion of polyploid cells and aberrant mitosis(Reddel *et al.* 1985; Basset *et al.* 1986; Hoyes *et al.* 1992). However, in this study these changes were not observed. The mechanism of the increased S phase by DFO can be considered in two ways; one is a block in the S phase and the other is a relative increase of S phase cells by selective death of G2/M or G0/G1 phase cells which might have been more sensitive to DFO. But the percent viability of Hep 3B cells was high (above 90%) even after up to 96 hours of incubation with 60 μg/ml of DFO, suggesting minimal cell death while the S phase increased, so the former was favored, and like the report of Hedley *et al.* (1985), it is believed that DFO caused the S phase accumulation by a block in the S phase with unbalanced cell growth, inhibition of cell proliferation and ensuing cell death. However, the synchronization experiment is required to assess which phase cells are selectively killed.

Although routine flow cytometry using PI demonstrated that the cell cycle effect of DFO on hepatoma cell lines was mainly a

dose dependent S phase increase, more studies were required to further characterize the block in the S phase and to understand the mechanism in view of tumor cell kinetics. The bivariate analysis using anti-BrdU antibody was performed to analyze the effects of DFO on the cancer cell kinetics of hepatoma cell lines. BrdU is a thymidine analogue that is incorporated specifically into DNA during the S phase of the cell cycle (Fujikane *et al.* 1989). In 1982, Gratzner produced a monoclonal anti-BrdU antibody, and, in 1983, Dolbeare *et al.* developed a procedure for simultaneous flow cytometric measurement of the cellular DNA content and the amount of incorporated BrdU. The BrdU labeling technique offers the advantage of simple and safe handling and a comparatively short processing time over [^{3}H] thymidine autoradiography in which use is limited by the exposure time(minimum 2 days) and technical complications connected with the use of radioactive substances(Langer *et al.* 1985). Recently it has been used for the research of cancer cell kinetic perturbation by chemotherapeutic agent (Fujikane *et al.* 1989; Hemmer, 1990; Fogt *et al.* 1991; Ubezio *et al.* 1991; Dermarcq *et al.* 1992) or radiotherapy (McNally and Wilson 1986). In 1985, Begg *et al.* introduced a method of estimating DNA synthesis time, Ts, using the relative movement(RM) of the labeled cells between the G0/G1 and G2/M phase after pulse labeling with BrdU. In this study, after BrdU pulse labeling, DFO caused an accumulation of mainly early S phase cells among early, mid, and late S -phases, and BrdU labeled cells moved little toward the mid or late-S phase with time progress. This finding suggests that the block site was in the mid-S phase or early-mid interface. Ts of DFO treated cells was markedly prolonged(54.1 hours), while BrdU labeled control cells progressed toward the G2/M phase with time and Ts was 9.9 hours.

The inhibitory effects of DFO on the DNA synthesis and effect on the cell cycle have been known to be reversible by washing or by adding the stoichiometric amount of Fe^{3+} (Lederman *et al.* 1984; Reddel *et al.* 1985; Basset *et al.* 1986; Tomoyasu *et al.* 1987; Becton and Roberts 1989; Blatt *et al.* 1989), and this reversibility of the antiproliferative effect of DFO can be a merit for DFO to be used as a chemotherapeutic agent(Nocka and Pelus, 1988). In this study, the action of DFO was stopped by a change of the culture medium to a DFO free medium. After the removal of DFO, the bivariate analysis showed a continued increase of the S phase and a decrease of the G0/G1 phase at 24 hours after release. However, when the S phase was more specifically divided into early, mid, and late-S phases, a sequential increase of the mid-S, late-S and G2/M phases was observed, which suggested that the blocked cells at the early-mid S phase or mid S phase were released to the mid-S and late-S phase in a partial synchronized manner. At 48 hours after the removal of DFO, the figure of the cell cycle was restored to that of the control(not described in result), which suggested the effect of DFO on the cell cycle of the hepatoma cells was reversible by the medium change, although time is required.

The iron chelator has been shown to be used as a cell cycle synchronization agent, and this has led to the suggestion that the iron chelator may be used to obtain a population of synchronized proliferating cells with enhanced sensitivity to cell cycle-specific antitumor agents (Bergeron and Ingeno 1987; Hoyes *et al.* 1992). In this study, the cell cycle of the hepatoma cells was blocked in the mid-S or early-mid interface and after the removal of DFO, the cells moved in a partial synchronized manner. This can explain the reported synergistic effect of DFO and S phase-dependent cell cycle specific agents (ARA-C and 5-FU) in leukemic cells (Estrov *et al.* 1987) and hepatoma cells (Song 1992).

In summary, this study indicated that the antiproliferative mechanism of DFO in human hepatoma cell lines was caused by the inhibitory effect of DNA synthesis, which was related to a block in the early-mid S interface or mid-S phase. But, in order to further characterize its mechanism, more studies including in vivo experiment are needed.

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