Flow Cytometric Analysis of the Cell Cycle of the Leukemic Cell Lines Treated with Etoposide and Cytosine Arabinoside

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ISHIYAMA, K., SATOH, S., IGARASHI, Y., KUMAGAI, H., YAHAGI, A. and SASAKI, H. *Flow Cytometric Analysis of the Cell Cycle of the Leukemic Cell Lines Treated with Etoposide and Cytosine Arabinoside.* Tohoku J. Exp. Med., 1994, 174 (2), 95-107 — Effects of etoposide (VP-16) and cytosine arabinoside (Ara-C) on the cell cycle of HL-60 and THP-1 cells were studied by flow cytometry using the bromodeoxyuridine (BrdU)/DNA assay technique to investigate the efficacy of VP-16 for monocytic leukemia cells. VP-16 inhibited the proliferation of THP-1 cells more strongly than that of HL-60 cells at any concentrations used at 24 and 48 hr. VP-16 arrested HL-60 and THP-1 cells in the G₂/M phase and reduced them in the G₀/G₁ and early S phase at higher concentrations. There was no significant difference in the percentage of G₂/M phase cells at the same concentration between both cells. However, reduction in the G₀/G₁ and early S phase cells was more marked in THP-1 than HL-60 cells significantly. On the other hand, Ara-C perturbed the cell cycle of HL-60 cells more than that of THP-1 cells at 24 and 48 hr. These results suggest that the effects of VP-16 on the cell cycle may be more intense in THP-1 than HL-60 cells, and support the efficacy of VP-16 for treating monocytic leukemia in vivo. ————flow cytometry; cell cycle; bromodeoxyuridine (BrdU); etoposide (VP-16); cytosine arabinoside (Ara-C)

Etoposide (VP-16), a newly developed semisynthetic derivative of podophyllotoxin (Loike and Horwitz 1976; Roberts et al. 1980), is recently available for the treatment of acute leukemias and malignant lymphomas, and has been found useful for relapsed or refractory cases (Mathé et al. 1974; Bishop et al. 1990). This drug is considered more effective for patients with monocytic leukemia than for those with other types of leukemia (Mathé et al. 1974; Bernasconi et al. 1982;

Received March 30, 1994; revision accepted for publication August 11, 1994.

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Abbreviations used in this paper: BrdU, bromodeoxyuridine; PI, propidium iodide; DNA, deoxyribonucleic acid; Ara-C, cytosine arabinoside; AMoL, acute monocytic leukemia; APL, acute promyelocytic leukemia; FCS, fetal calf serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; s.e., standard error.

Varini and Cavalli 1982; Odom and Gordon 1984; Moriyama et al. 1985). However, its efficacy for monocytic leukemia cells has yet to be determined.

VP-16 causes G₂ block in the cell cycle by flow cytometric analysis using propidium iodide (PI) staining (Krishan et al. 1975). A new method for flow cytometric analysis of the cell cycle using the anti-bromodeoxyuridine (BrdU) monoclonal antibody, which was previously created (Gratzner 1982), and PI staining was developed (Dolbeare et al. 1983). This method needs no special facilities nor special technical skills like other methods using radioisotopes and has been applied to many clinical studies on the cell cycle (Katano et al. 1989; Tafuri and Andreeff 1990; Bettelheim et al. 1991). So we studied effects of antileukemic agents on the cell cycle of leukemic cells using this method to investigate the efficacy of VP-16 for monocytic leukemia cells in vitro.

In this study, we used two human leukemic cell lines, THP-1 cells, a human leukemic cell line derived from the peripheral blood of a patient with acute monocytic leukemia (AMoL) (Tsuchiya et al. 1980), and HL-60 cells, a human leukemic cell line derived from the peripheral blood of a patient with acute promyelocytic leukemia (APL) (Collins et al. 1977). The effects of VP-16 on the cell cycle of both cells were examined by flow cytometric analysis using the anti-BrdU monoclonal antibody/PI assay technique and compared between both of the cells to find that this method could detect the efficacy of VP-16 for monocytic leukemia cells in vitro. The effects of cytosine arabinoside (Ara-C) on the cell cycle of both cells were also examined and compared with those of VP-16.

MATERIALS AND METHODS

Antileukemic agents. VP-16 was kindly provided by Nippon Kayaku Co., Ltd. (Tokyo) and Ara-C, by Nippon Shinyaku Co., Ltd. (Tokyo).

Cell culture. HL-60 cells were kindly provided by Dr F. Sendo (Department of Parasitology and Immunology, Yamagata University, Yamagata) and THP-1 cells, by Dr S. Yokoyama (Department of Pediatrics, Yamagata University, Yamagata). HL-60 and THP-1 cells were grown in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% fetal calf serum (FCS) (Bioproducts, Inc., Walkersville, MD, USA) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C and maintained by dilution with fresh medium twice weekly.

Exponentially growing HL-60 and/or THP-1 cells were suspended in 10 ml of RPMI 1640 medium containing 10% FCS at a density of 2×10^5 cells/ml in 25-cm² tissue culture flasks (Sumilon, Sumitomo Bakelite Co., Ltd., Tokyo) and incubated with or without antileukemic agents (VP-16 or Ara-C) at various concentrations for 24 or 48 hr. Viable cells were counted by the trypan blue dye exclusion method at the end of culture.

Staining. Staining procedure was performed according to the modified method which was reported previously (Dolbeare et al. 1983). Briefly, BrdU (Sigma Chemical Co., St. Louis, MO, USA) was added to the medium at a final

concentration of $5\text{ }\mu\text{g/ml}$ 30 min before the end of culture. The cells were harvested and washed twice with cold phosphate-buffered saline (PBS) by centrifugation at 4°C and fixed with 2 ml of 70% ethanol for more than 30 min. DNA of ethanol-fixed cells (2×10^6 cells) was partially denaturated by exposure to 1 ml of 4N HCl at room temperature for 20 min. The cells were neutralized by 0.1 M sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$) (Wako Chemical Co., Ltd., Osaka), washed with PBS and resuspended in $50\text{ }\mu\text{l}$ of PBS containing 0.5% Tween 20 (Serva Co., Heidelberg, NY, USA). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (Becton-Dickinson Co., Ltd., San Jose, CA, USA) in the dark at room temperature for 30 min. The cells were washed twice with PBS by centrifugation and resuspended in PBS. Cellular double-stranded DNA was stained with PI (Sigma Chemical Co.) at $50\text{ }\mu\text{g/ml}$ for 15 min. Clumps of cells were then removed by filtration through $50\text{-}\mu\text{m}$ nylon mesh and the filtered cells were analyzed by a flow cytometer.

Flow cytometry. The stained cells were analyzed by a flow cytometer, FACStar (Becton-Dickinson Co., Ltd.). The excitation wavelength was 488 nm and the fluorescent wavelengths of FITC and PI were 530 and 575 nm, respectively. Routinely, 10,000 cells were measured for each distribution at a flow rate of less than 300 cells/sec. Bivariate BrdU/DNA distribution (64×64 channel) was obtained by software Consort 30 program (Becton-Dickinson Co.). Fig. 1 shows the bivariate BrdU/DNA distribution of exponentially growing HL-60 cells. It was plotted as a contour graph by the cell number of each channel. Windows for

Fig. 1. Bivariate BrdU/DNA distribution of exponentially growing HL-60 cells. Distribution is shown for untreated control cells. Four windows indicate phases of the cell cycle.

the cells in each phase of the cell cycle were set as indicated in Fig. 1. Cells with low DNA content and low BrdU incorporation were defined as those in the G_0/G_1 phase, cells with twice the DNA content and low BrdU incorporation as in the G_2/M phase and those with high BrdU incorporation as in the S phase. The S phase was divided into early and late S subphases by the division line between G_0/G_1 and G_2/M phases.

Statistics. Results were expressed as the means $\pm$ s.e. All p values were obtained by the non-paired t test.

Fig. 2. Relative cell counts of HL-60 and THP-1 cells after the treatment with VP-16. HL-60 (■) or THP-1 (▨) cells were incubated with or without VP-16 at various concentrations for 24 (A) or 48 hr (B). Viable cells were counted by the trypan blue dye exclusion method at the end of culture. Viable cell counts of treated cells are indicated as the percent of untreated control cells. Each value represents the mean of 4 separate experiments. Bars represent s.e. * $p < 0.05$ (HL-60 vs. THP-1 cells).

RESULTS

Effects of VP-16 on the cell cycle of HL-60 and THP-1 cells. VP-16 inhibited the proliferation of HL-60 and THP-1 cells at 24 and 48 hr in a dose-dependent manner (Fig. 2). Inhibition of the proliferation was stronger in THP-1 than HL-60 cells at 24 and 48 hr (significant at 100 ng/ml at both 24 and 48 hr; $p < 0.05$, $p < 0.05$, respectively) (Fig. 2).

Representative flow cytograms of HL-60 and THP-1 cells treated with VP-16 at a concentration of 100 ng/ml for 24 hr are shown in Fig. 3. VP-16 caused G_2 block in the cell cycle of both cells. The cell cycle of each cell at 24 hr is shown in Fig. 4. The number indicates the percentage of cells in each phase of the cell cycle. In HL-60 cells, VP-16 caused relative increase in the G_2/M phase cells at 100 ng/ml, and also caused reduction in the G_0/G_1 and early S phase cells at the

Fig. 3. Bivariate BrdU/DNA distributions of HL-60 and THP-1 cells after the treatment with VP-16. Representative flow cytograms of HL-60 (A) and THP-1 cells (B) treated with VP-16 at a concentration of 100 ng/ml for 24 hr are shown.

Fig. 4. Cell cycle of HL-60 and THP-1 cells after the treatment with VP-16 for 24 hr. HL-60 (●) or THP-1 cells (○) were incubated with or without VP-16 at various concentrations for 24 hr. Relative cell counts of treated and untreated cells in each phase of the cell cycle at 24 hr were analyzed by flow cytometry using the anti-BrdU/DNA assay technique. Each value represents the mean and s.e. of 4 separate experiments. * $p < 0.05$ (treated vs. untreated cells); ** $p < 0.01$; † $p < 0.05$ (HL-60 vs. THP-1 cells); †† $p < 0.01$.

same concentration. In THP-1 cells, relative increase in the G_2/M phase cells was observed at 10 and 100 ng/ml, which was more marked than in HL-60 cells (although there were no significant differences). Reduction in the G_0/G_1 and early S phase cells was also detected at 10 and 100 ng/ml, and was more marked in THP-1 than HL-60 cells (significant in the G_0/G_1 phase cells at 100 ng/ml, $p < 0.01$; the early S phase cells at 10 ng/ml and 100 ng/ml, $p < 0.05$, $p < 0.01$, respectively). Relative increase in the late S phase cells was observed at 100 ng/ml in THP-1 cells only. In the cell cycle at 48 hr (Fig. 5), relative increase in the G_2/M phase cells was detected at 10 or 100 ng/ml in both cells, but there were no significant differences in the percentage of the G_2/M phase cells between both cells. Reduction in the early and late S phase cells at 100 ng/ml was more marked in THP-1 than HL-60 cells (significant in the early S phase cells, $p < 0.01$; the late S phase cells, $p < 0.05$).

Effects of Ara-C on the cell cycle of HL-60 and THP-1 cells. Ara-C inhibited the proliferation of HL-60 and THP-1 cells in a dose-dependent manner, as noted for VP-16 (Fig. 6). At 24 hr, Ara-C inhibited the proliferation of HL-60 more than THP-1 cells at 1 and 10 ng/ml ($p < 0.05$, $p < 0.05$, respectively) (Fig. 6-A). At 48 hr, cell proliferation was inhibited more in THP-1 than HL-60 cells

Fig. 5. Cell cycle of HL-60 and THP-1 cells after the treatment with VP-16 for 48 hr. HL-60 (●) or THP-1 cells (○) were incubated with or without VP-16 at various concentrations for 48 hr. Relative cell counts of treated and untreated cells in each phase of the cell cycle at 48 hr were analyzed by flow cytometry using the anti-BrdU/DNA assay technique. Each value represents the mean and s.e. of 4 separate experiments. * $p < 0.05$ (treated vs. untreated cells); ** $p < 0.01$; † $p < 0.05$ (HL-60 vs. THP-1 cells); †† $p < 0.01$.

at 0.1 ng/ml ($p < 0.05$), in HL-60 than THP-1 cells at 10 ng/ml ($p < 0.05$) (Fig. 6-B).

Representative flow cytograms of HL-60 and THP-1 cells treated with Ara-C at a concentration of 100 ng/ml for 24 hr are shown in Fig. 7. In the cell cycle at 24 hr (Fig. 8), Ara-C caused relative increase in the early S phase cells and reduction in the G₀/G₁ phase cells at 10 ng/ml in HL-60 cells, but not in THP-1 cells (significant in the early S and G₀/G₁ phase cells: $p < 0.05$, $p < 0.05$, respectively). On the other hand, reduction in the early and late S phase cells and relative increase in the G₀/G₁ phase cells were observed in HL-60 cells at 100 ng/ml, although relative increase in the early S phase cells and reduction in the G₀/G₁ phase cells were still seen in THP-1 cells at the same concentration (significant in the G₀/G₁ phase cells, $p < 0.01$; the early and late S phase cells, $p < 0.01$, $p < 0.01$, respectively). In the cell cycle of 48 hr (Fig. 9), relative increase in the early S phase cells was observed at 1 and 10 ng/ml in HL-60 cells only. Reduction in the G₀/G₁ phase cells was observed at the same concentrations in both of the cells and was more marked in HL-60 than THP-1 cells. Relative increase in the G₀/G₁ phase cells at 100 ng/ml was also observed in HL-60 cells only.

Fig. 6. Relative cell counts of HL-60 and THP-1 cells after the treatment with Ara-C. HL-60 (■) or THP-1 (▨) cells were incubated with or without Ara-C at various concentrations for 24 (A) or 48 hr (B). Viable cells were counted by the trypan blue dye exclusion method at the end of culture. Viable cell counts of treated cells are indicated as the percent of untreated cells. Each value represents the mean of 4 separate experiments. Bars represent s.e. * $p < 0.05$ (HL-60 vs. THP-1 cells).

DISCUSSION

VP-16 is a recently developed anticancer agent belonging to the DNA topoisomerase II inhibitor and exerts strong antileukemic activity by inhibiting the synthesis of DNA in the S phase cells and inducing single-stranded breaks of DNA (Loike and Horwitz 1976; Roberts et al. 1980). In the analysis of the cell cycle by PI staining, VP-16 arrests leukemic cells in the G_2 phase (Krishan et al. 1975). The mechanism of the G_2 block by VP-16 is considered to be as follows: cells whose DNA has been damaged in the S phase by VP-16 pass in some manner through the S phase, but need much more time in the G_2 phase to repair the

Fig. 7. Bivariate BrdU/DNA distributions of HL-60 and THP-1 cells after the treatment with Ara-C. Representative flow cytograms of HL-60 (A) and THP-1 cells (B) treated with VP-16 at a concentration of 100 ng/ml for 24 hr are shown.

damaged DNA (Charcosset 1986). In this study, relative increase in the G_2/M phase cells was observed by the treatment with VP-16 with the concentration of 100 ng/ml in THP-1 and HL-60 cells. Since the plasma level of VP-16 obtained from a patient treated with a standard dose therapy of this drug is about 1 to 10 mg/ml (Wakui et al. 1986), the perturbation of the cell cycle could be revealed by VP-16 of much lower concentrations than those obtained by clinical doses. There were no significant differences in relative increase in the G_2/M phase cells between both of the cells, but reduction in the G_0/G_1 and early S phase cells was more marked in THP-1 than HL-60 cells. So the perturbation of the cell cycle by VP-16 was more remarkable in THP-1 than HL-60 cells. VP-16 also inhibited the proliferation of THP-1 cells more strongly than that of HL-60 cells at the same concentrations. According to these observations, the effects of VP-16 on the cell cycle would be more intense in THP-1 than HL-60 cells. These results are

Fig. 8. Cell cycle of HL-60 and THP-1 cells after the treatment with Ara-C for 24 hr. HL-60 (●) or THP-1 cells (○) were incubated with or without Ara-C at various concentrations for 24 hr. Relative cell counts of treated and untreated cells in each phase of the cell cycle at 24 hr were analyzed by flow cytometry using the anti-BrdU/DNA assay technique. Each value represents the mean and s.e. of 4 separate experiments. * $p < 0.05$ (treated vs. untreated cells); ** $p < 0.01$; † $p < 0.05$ (HL-60 vs. THP-1 cells); †† $p < 0.01$.

compatible with the finding that VP-16 is more effective for patients with monocytic leukemia than for those with other types of leukemia (Mathé et al. 1974; Bernasconi et al. 1982; Varini and Cavalli 1982; Odom and Gordon 1984; Moriyama et al. 1985). The present in vitro data would support the efficacy of VP-16 for treating monocytic leukemia in vivo.

Ara-C is a type of antimetabolite agent and the key drug for treating acute non-lymphocytic leukemia. Its effect is specific for cells in the S phase. Analysis of the cell cycle has shown Ara-C to cause S delay at lower concentrations and G_1 -S block at higher concentrations (Charcosset 1986). In this study, Ara-C arrested HL-60 cells in the early S phase at lower concentrations (1 and 10 ng/ml). The peak plasma concentration obtained from a patient after subcutaneous injection of low dose Ara-C is about 50 to 100 ng/ml (Ishikura et al. 1984), and the plasma concentration obtained by continuous infusion of standard dose Ara-C is about 10 to 100 ng/ml (Hirschmann et al. 1968). So the perturbation of the cell cycle by Ara-C revealed at much lower concentrations than those obtained by clinical doses. Ara-C arrested HL-60 cells in the G_0/G_1 phase at higher concentration (100 ng/ml), which could be obtained by a standard or high dose therapy of Ara-C (Hirschmann et al. 1968; Urabe et al. 1985). Relative increase in the early

Fig. 9. Cell cycle of HL-60 and THP-1 cells after the treatment with Ara-C for 48 hr. HL-60 (●) or THP-1 cells (○) were incubated with or without Ara-C at various concentrations for 48 hr. Relative cell counts of treated and untreated cells in each phase of the cell cycle at 48 hr were analyzed by flow cytometry using the anti-BrdU/DNA assay technique. Each value represents the mean and s.e. of 4 separate experiments. * $p < 0.05$ (treated vs. untreated cells); ** $p < 0.01$; † $p < 0.05$ (HL-60 vs. THP-1 cells); †† $p < 0.01$.

S phase cells and reduction in the G₀/G₁ phase cells at lower concentration was more marked in HL-60 than THP-1 cells. Relative increase in the G₀/G₁ phase cells at higher concentration was observed in HL-60 cells only. Ara-C may thus be more effective for HL-60 than THP-1 cells in vitro. But there is no correlation between the cell cycle perturbation and the growth inhibition, so the specificity of Ara-C for cell type is not so great as that of VP-16.

The present results could not provide general conclusions because only one type of monocytic leukemia cells was examined, but they would indicate that VP-16 is effective particularly for monocytic leukemia in vitro as well as in vivo. Since flow cytometric analysis of the cell cycle by the BrdU/DNA assay technique could detect differences of the perturbation of the cell cycle by antileukemic agents between both of the cells, it may be useful for a chemosensitivity test of antileukemic agents. We are examining further on leukemic cells from clinical patients.

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