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ORIGINAL ARTICLE

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Effect of the exposure dose of etoposide on the cell growth and cell kinetics of human ovarian cancer cells

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Abstract Although schedule-dependent cytotoxicity of etoposide has been reported, its mechanisms have not been elucidated fully. In this study, we attempted to clarify what concentration, time or exposure dose (ED, concentration of drug × time) of etoposide result in the antitumor effect on human ovarian cancer cells from the standpoint of cell cycle perturbation. The different ED were produced by different drug treatment schedules: 10 μg/ml × 4 h (ED 40), 1.66 μg/ml × 24 h (ED 40), 5 μg/ml × 4 h (ED 20), 0.83 μg/ml × 24 h (ED 20), 10 μg/ml × 0.8 h (ED 8), 5 μg/ml × 1.6 h (ED 8), 2 μg/ml × 4 h (ED 8), 0.33 μg/ml × 24 h (ED 8). Cell cycle perturbation on day 5 and the suppression of cell growth were dependent on the drug concentration at the lowest exposure dose (ED 8), but were dependent on ED at the higher EDs (20 or 40). The percentage of cells in the G_2/M fraction significantly decreased from day 5 to day 7 in BG-1 cells treated at ED 20 or treated with higher concentrations (10 and 5 μg/ml) at ED 8, but not in those treated at ED 40 or treated with lower concentrations (2 and 0.33 μg/ml) at ED 8. Therefore, the cytotoxic mechanism of etoposide may not be explained by simple schedule dependency.

Key words Etoposide · Cell kinetics · Exposure dose

Introduction

The glycosidic derivative of epipodophyllotoxin, etoposide, exhibits a wide spectrum of antineoplastic activity and is increasingly used in the treatment of cancer. This agent induces an irreversible blockade of cells in the premitotic phase of the cell cycle, leading to accumulation of cells in the late S or G_2 phases [1, 8]. Its cytotoxic effects appear to result from single- and double-strand breaks in DNA and DNA–protein crosslinks [7, 13]. Dombernowsky and Nissen first reported the schedule dependency of the antileukemic activity of etoposide against the L1210 ascites tumor in N/D mice [2]. They demonstrated that daily treatment for 5 days was superior to treatment on day 1 only providing that the total amount of etoposide was equivalent. Slevin et al. also reported other clinical evidence of the importance of drug scheduling for etoposide [11]. In their study, they found that treatment f previously untreated extensive small-cell lung cancer patients with etoposide, with five consecutive daily 2-h infusions, each of 100 mg/m^2, was superior to 500 mg/m^2 as a continuous intravenous infusion over 24 h even with similar areas under curve (AUC). However, the mechanisms governing the difference in these clinical responses by different administration schedules of etoposide have not been elucidated.

The AUC in vivo seems to correspond to the exposure dose (ED, concentration of drugs × time) in vitro. In this study, we attempted to clarify what concentration, time or ED of etoposide results in the antitumor effect on human ovarian cancer cells from the standpoint of cell cycle perturbation.

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Materials and methods

Cells

BG-1 cells, derived from a human ovarian carcinoma [3, 6], were used in this study. These cells were maintained in McCoy's Medium 5A supplemented with 10% fetal bovine serum, 0.05% L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Exponentially growing BG-1 cells were plated at 1×10^6 in 75-cm^2 culture flasks with 15 ml medium. The cells were incubated in a 100% humidified atmosphere containing 5% CO_2 at 37°C.

Etoposide treatment

The etoposide, purchased from Bristol-Myers-Squibb K.K., Japan, was prepared shortly before treatment and diluted to ten times the desired concentrations with phosphate-buffered saline. On day 3, 1.65 ml of drug was added to each flask. The final concentrations were 10, 5, 2, 1.66, 0.83, and 0.33 μg/ml. After the designated times, the drug treatments were terminated by replacing the drug-containing medium with fresh medium. Etoposide treatment was calculated to give EDs of 40 (10 μg/ml × 4 h, 1.66 μg/ml × 24 h), 20 (5 μg/ml × 4 h, 0.83 μg/ml × 24 h) and 8 (10 μg/ml × 0.8 h, 5 μg/ml × 1.6 h, 2 μg/ml × 4 h, 0.33 μg/ml × 24 h). Nuclear isolation was performed on either day 5 or day 7.

Growth curve

The cells on day 5 and day 7 were removed from the flasks with 0.1% trypsin and the cell number for each treatment was scored.

Nuclear isolation

The nuclei of the BG-1 cells were isolated using a previously described method [4]. Briefly, the cells were removed from the flasks with 0.1% trypsin and resuspended in the medium at approximately 3×10^6. Then they were washed three times with spinner salts (5.4 m*M* KCl, 0.4 m*M* $MgSO_4$, 0.12 *M* NaCl, 26 m*M* $NaHCO_3$, 10 m*M* NaH_2PO_4, 5.5 m*M* D-glucose). Next, they were resuspended in a Triton X-100 solution (1% Triton X-100 in 0.08 *M* NaCl, 0.02 *M* EDTA) and incubated for 10 min to remove the cytoplasm. If visual inspection revealed no significant cytoplasmic contamination, the nuclei were then washed with 0.15 *M* NaCl and resuspended with propidium iodide (PI). The entire isolation procedure was performed at an ice-cold temperature.

DNA analysis

The nuclei were stained with 50 μg/ml PI to determine the DNA content, and then were held overnight at an ice-cold temperature. Analysis was performed on a FACScan flow cytometer (Becton-Dickinson) with a 488 nm excitation beam. Three parameters were measured: forward-angle light scatter, side-angle light scatter, and PI fluorescence (red, 610 nm). The percentages of cells in G_1, S and G_2/M phases were analyzed using a cell cycle analysis program (Consort 30).

Statistical analysis

The results are shown as means ± SE, and statistical significance was determined using Student's *t*-test.

Results

Figure 1 shows growth curves of BG-1 cells after etoposide treatment. Untreated BG-1 cells grew exponentially until day 5, after which growth reached a plateau. When the cells were treated with etoposide on day 3, the growth of the cells was suppressed. The growth of the cells seemed to be dependent on the exposure dose at ED 40 or ED 20 despite the difference in treatments, but at ED 8 the suppression of growth was dependent on the concentration of etoposide.

Representative DNA histograms of the BG-1 cells with or without etoposide treatment on day 5 are shown in Fig. 2. The percentage of cells in G_2/M phase in untreated day-5 cells was 25.2 ± 1.6%. In comparison with the control, all treatments induced a pronounced G_2/M block. The percentage of cells in G_2/M phase at ED 40 and 20 appeared to be dependent on the ED despite the different concentrations and different exposure times. The percentage of cells in G_2/M phase in cells treated with 10 μg/ml × 0.8 h appeared to be higher than with the other three concentrations at ED 8. Figure 3 shows the percentage of day-5 BG-1 cells in G_2/M phase with eight different treatments. When the percentage of cells in G_2/M phase was compared, a significant difference was observed between each of the ED treatments. The percentage of cells in G_2/M phase did not differ in the ED 40 and 20 groups with different concentrations and times of

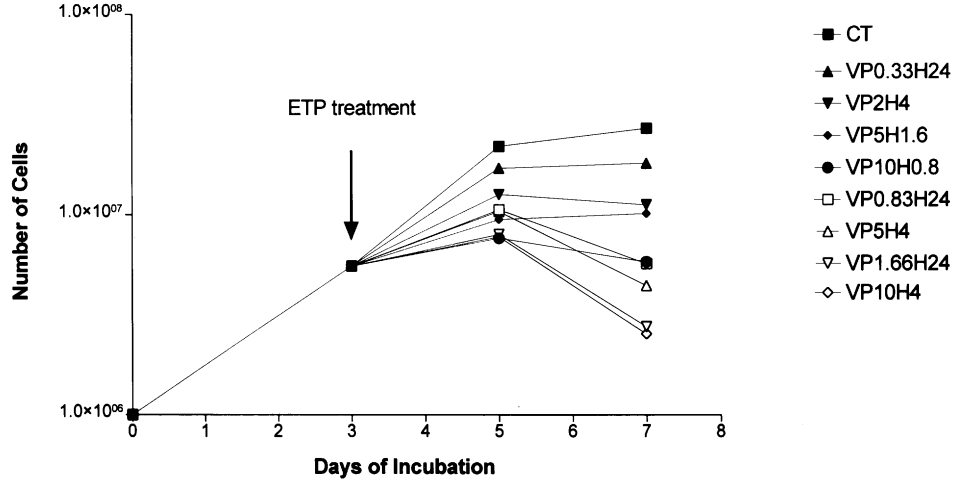

Fig. 1 Growth of BG-1 cells with or without etoposide treatment (■ untreated; ED 8: ▲ 0.33 μg/ml × 24 h, ▼ 2 μg/ml × 4 h, ◆ 5 μg/ml × 1.6 h, ● 10 μg/ml × 0.8 h; ED 20: □ 0.83 μg/ml × 24 h, △ 5 μg/ml × 4 h; ED 40: ▽ 1.66 μg/ml × 24 h, ◇ 10 μg/ml × 4 h)

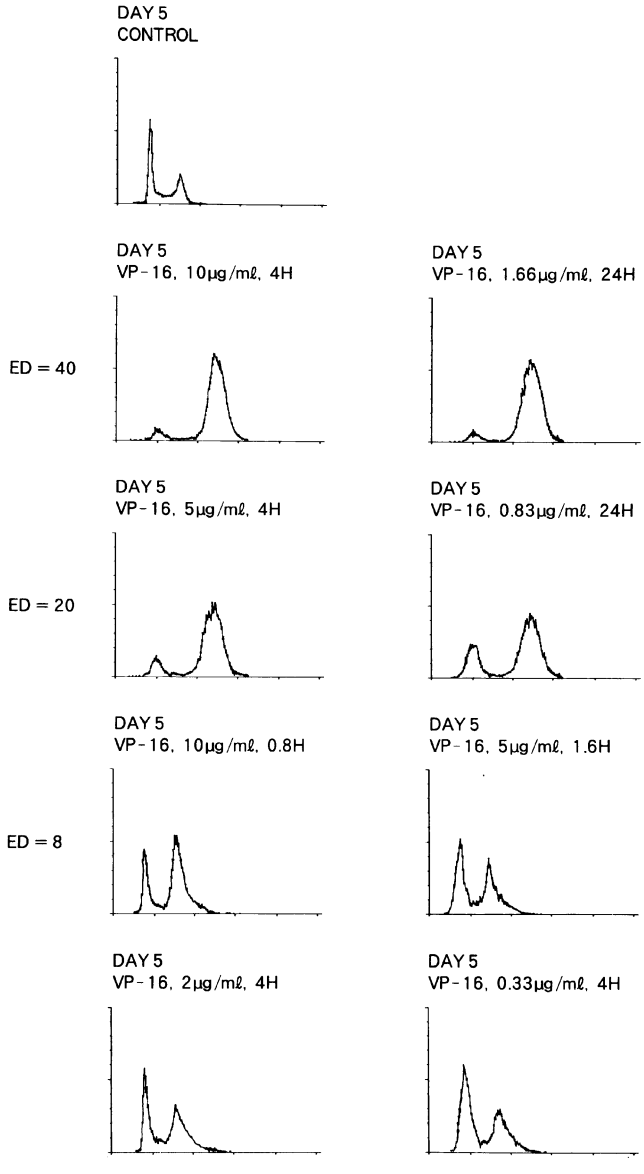

Fig. 2 Ungated DNA histograms of BG-1 cells on day 5

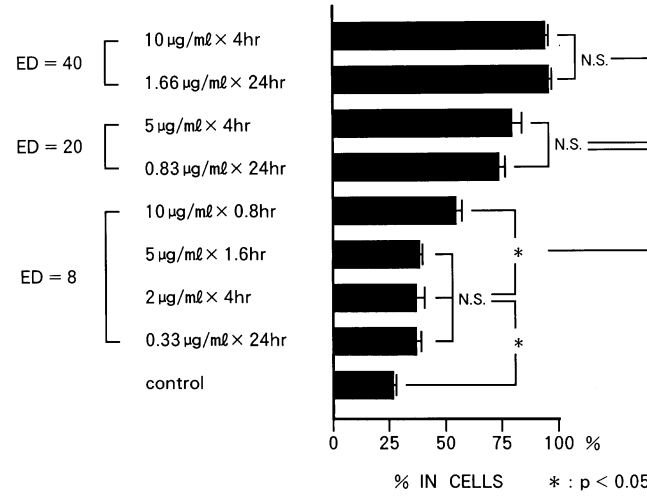

Fig. 3 The percentage of day-5 BG-1 cells in G_2/M phase-was 90.7 ± 0.7% with etoposide at 10 μg/ml × 4 h, 92.7 ± 0.3% at 1.66 μg/ml × 24 h, 77.3 ± 4.5% at 5 μg/ml × 4 h, 71.3 ± 1.8% at 0.83 μg/ml × 24 h, 52.8 ± 2.8% at 10 μg/ml × 0.8 h, 37.8 ± 1.5% at 5 μg/ml × 1.6 h, 35.1 ± 2.8% at 2 μg/ml × 4 h and 35.4 ± 1.9% at 0.33 μg/ml × 24 h (*NS* not significant; $*P < 0.05$)

exposure to etoposide, but differences in the percentage of cells in G_2/M phase between 10 μg/ml × 0.8 h and the other three lower concentrations at ED 8 were observed, suggesting that lower concentrations at low ED of etoposide could not induce as large a G_2/M block as other treatments.

Representative DNA histograms of BG-1 cells with or without etoposide treatments on day 7 are shown in Fig. 4. The percentage of cells in G_2/M phase is shown in Fig. 5. Similar results were obtained in day-7 BG-1 cells as in day-5 cells. At this time, no significant differences in the percentages of cells in G_2/M phase were observed between the ED 20 treatments and the 10 μg/ml × 0.8 h treatment at ED 8.

We compared the percentage of cells in G_2/M phase on day 5 with that on day 7 for each treatment. When the BG-1 cells were treated with etoposide at ED 40 there were no significant differences in the percentage of cells in G_2/M phase between day 5 and day 7 either at 10 μg/ml × 4 h or 1.66 μg/ml × 24 h (Fig. 6). However, the percentage of cells in G_2/M phase from day 5 to day 7 was significantly different at ED 20 regardless of the treatment schedule (Fig. 7). At ED 8, the difference was significant when the cells were treated with 10 μg/ml × 0.8 h and 5 μg/ml × 1.6 h, but not significant when they were treated with 2 μg/ml × 4 h and 0.33 μg/ml × 24 h (Fig. 8)

Discussion

The primary purpose of this study was to elucidate the relationship between the cytotoxic effect of etoposide and its ED, which corresponds to AUC in vivo, from the standpoint of cell kinetics.

The cell cycle data of day 5 indicated that the percentage of cells in G_2/M phase was dependent on ED. There were no differences in the percentages of cells in G_2/M phase between two different drug treatments at ED 40 and 20, whereas they were significantly different between treatments with 10 μg/ml and the other three lower concentrations at ED 8. These findings suggest that when cells are exposed to certain EDs such as ED 20 or 40 the cytotoxicity of etoposide is dependent on the ED. However, the drug concentration is an important factor at lower EDs. The difference in the cell cycle perturbations at ED 8 with different schedules was also seen in the cell growth curves. However, the suppression of cell growth was dose-dependent.

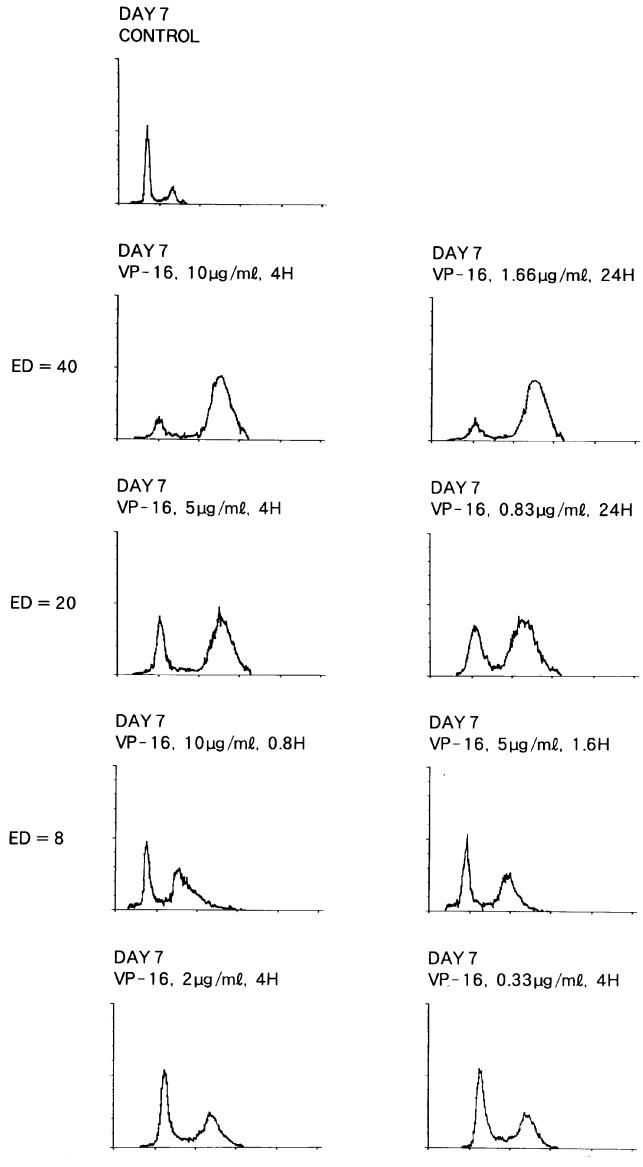

Fig. 4 Ungated DNA histograms of BG-1 cells on day 7

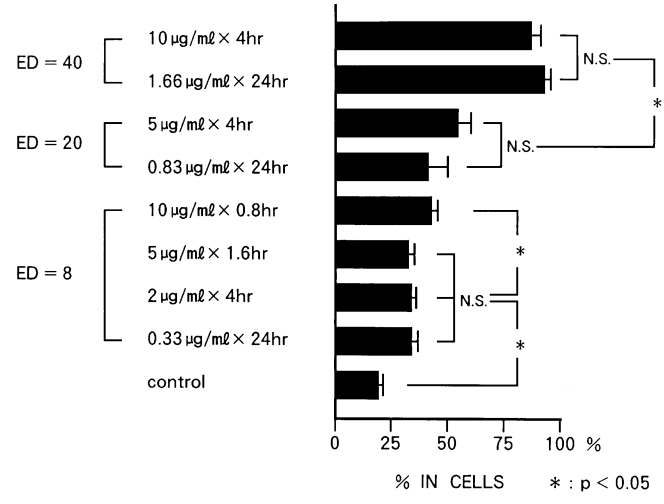

Fig. 5 The percentage of day-7 BG-1 cells in G_2/M phase was $84.3 \pm 3.8\%$ with etoposide at 10 μg/ml × 4 h, $89.7 \pm 2.4\%$ at 1.66 μg/ml × 24 h, $54.3 \pm 5.3\%$ at 5 μg/ml × 4 h, $41.3 \pm 7.9\%$ at 0.83 μg/ml × 24 h, $42.2 \pm 2.2\%$ at 10 μg/ml × 0.8 h, $31.6 \pm 2.2\%$ at 5 μg/ml × 1.6 h, $32.9 \pm 1.7\%$ at 2 μg/ml × 4 h and $32.8 \pm 2.5\%$ at 0.33 μg/ml × 24 h (*NS* not significant; $*P < 0.05$)

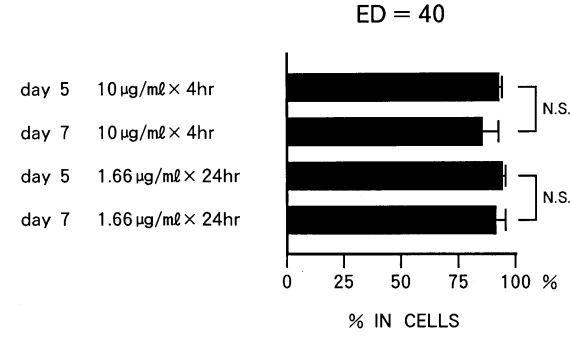

Fig. 6 Comparison of the percentage of BG-1 cells in the G_2/M phase between day 5 and day 7 at ED 40

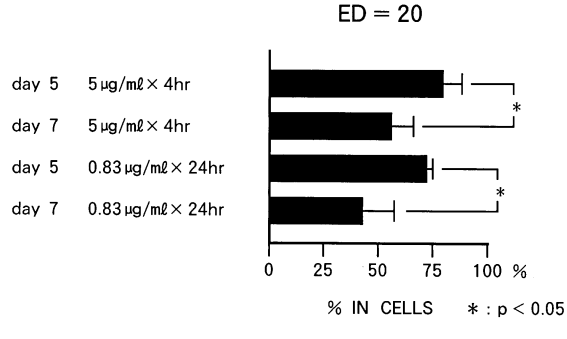

Fig. 7 Comparison of the percentage of BG-1 cells in the G_2/M phase between day 5 and day 7 at ED 20

These results partly support the suggestions made by Slevin et al. and Roed et al. [10, 12]. Slevin et al. suggested that the number of hours at concentrations of etoposide above 1 μg/ml was important. In this study, treatment with etoposide at, 0.83 μg/ml for 24 h and 1.66 μg/ml for 24 h produced equivalent cytotoxicity in terms of cell growth suppression and cell cycle perturbation to higher concentrations for shorter exposure times. In contrast to the previous studies which showed schedule-dependent cytotoxicity of etoposide, we did not find schedule dependency from the standpoint of cell cycle analysis. The suppression of cell growth and the effects on cell cycle perturbation by etoposide were dependent on ED at ED 40 and 20. These results indicate that the effect of etoposide would be AUC-dependent in vivo. This may be because the time of exposure to etoposide was too short in our study. Further investigation is required.

At ED 8, the percentages of cells in G_2/M phase at 10 μg/ml × 0.8 h and 5 μg/ml × 1.6 h seen on day 5

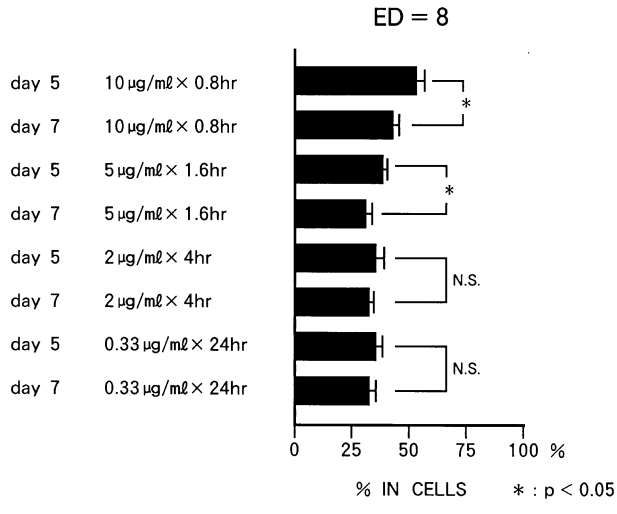

Fig. 8 Comparison of the percentage of BG-1 cells in the G_2/M phase between day 5 and day 7 at ED 8

disappeared on day 7. This suggests that the cells trapped in G_2/M phase after these treatments with etoposide easily started recycling because the damage was not lethal. This observation is supported by the study of Long et al. showing that changing to a drug-free medium after 1 h of drug exposure results in rapid exponential repair of both single- and double-strand DNA breaks [9]. The difference in the percentages of cells in G_2/M phase between 2 μg/ml × 4 h and 0.33 μg/ml × 24 h on day 5 and day 7 was not significant probably because the damage was too little. At ED 20, the percentage of cells in the G_2/M phase on day 5 either with 5 μg/ml × 4 h or 0.83 μg/ml × 24 h significantly decreased on day 7. However, there was no significant difference in the percentage of cells in G_2/M phase with ED 40 treatment between day 5 and day 7. These results suggest that the cytotoxicity of etoposide at ED 40 was so severe that nearly all cells were lethally damaged, whereas a portion of the cells at ED 20 started recycling because of less cytotoxicity. Again, we found ED dependency in the suppression of cell recycling.

In conclusion, the following results were found:

(1) Cell cycle perturbation and suppression of cell growth were dependent on ED when ED was either 20 or 40.
(2) Cell cycle perturbation and suppression of cell growth were dependent on the drug concentration at ED 8.
(3) The percentage of cells in G_2/M phase significantly decreased from day 5 to day 7 in cells treated at ED 20 and ED 8 with higher concentrations, but not at ED 40 and ED 8 with lower concentrations.

These observations could not explain the schedule-dependent cytotoxicity of etoposide found by Slevin et al. [11]. Since the schedule dependency of etoposide has been clinically observed, but not in our study, there are several factors that we have to consider when we interpret these results. The time of exposure to etoposide in this study was 24 h at most whereas it was several days in the clinical study. The population of cells in the cell cycle was also different between our study and the in vivo situation. The cells used in this study were exponentially growing, whereas a considerable proportion of cells in clinical cancers are quiescent. These factors significantly influence both the cell kinetics and clinical responses. Therefore, further experiments are required using longer exposure times and an in vivo model to acquire a better understanding of the schedule dependency of etoposide. Another important factor that should be taken in account in future studies is the endpoint combined with the cell-cycle study. We used change in the growth curve as a measure of growth suppression. Since growth retardation of the cells does not always reflect the cell kill, we have to examine the relationship between cell cycle perturbation and cell kill directly measured by an appropriate method such as a colony formation assay [5]. In spite of these questions to be elucidated in the future, this study has drawn an interesting relationship between cell kinetics and different dosing schedules of etoposide.

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