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Discrimination of Mitotic Cells Using Anti-p105 Monoclonal Antibody to Analyze the Mode of Action of Etoposide and Podophyllotoxin in Human Gastric Cancer Cells

Shigekazu Ohyama,[1] Yutaka Yonemura,[1] Kouichirou Tsugawa,[1] Itsuo Miyazaki,[1] Motohiro Tanaka[2] and Takuma Sasaki[2,3]

[1]*Department of Surgery II, School of Medicine, and* [2]*Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Takara-machi 13-1, Kanazawa 920*

Anti-p105 monoclonal antibody was used to discriminate between M-phase and G_2-phase of gastric cancer cells. p105 is a proliferation-associated nuclear antigen and its expression increases with cell cycle progression, especially in the mitotic phase. As an example of cell cycle analysis, the modes of action of etoposide and podophyllotoxin were examined by multiparameter flow cytometry. We found that etoposide caused G_2 block and retarded S phase transit and podophyllotoxin caused potential M phase block in gastric cancer cells. This cell cycle analysis by using anti-p105 monoclonal antibody should be useful for analysis of the actions of anti-tumor agents, especially for M phase analysis, because of its convenience and reliability.

Key words: Cell cycle analysis — Flow cytometry — Proliferation-associated nuclear antigen

One of the most common applications of flow cytometry has been the elucidation of the mode of action of anti-tumor agents. However, single parameter analysis based on the DNA histograms has an inherent limitation in terms of cell cycle analysis. So, several methods[1-5] for measurement of the fractions of cells in G_0G_1, S, and G_2M have been developed. For distinction of G_2M fractions, Darzynkiewicz *et al.*,[6] Roti *et al.*,[4] and Nusse *et al.*[2] reported several methods to distinguish G_2 cells from mitotic ones by using acridine orange (AO), fluorescein isothiocyanate, and bromodeoxyuridine, respectively. The first method is based on the differences in structure of nuclear chromatin as shown by staining with AO. The second used differences of nuclear protein fluorescence as a function of the cell cycle and the third used 90° and forward scatter measurements.

We present here a method to distinguish mitotic cells from G_2 phase cells by using a monoclonal antibody against p105. p105, a proliferation-associated nuclear antigen,[7] is expressed with cell cycle progression,[8] and dramatically increases in mitotic cells, in which its level is 5- to 10-fold greater than in G_2 cells.[9] We used the expression of p105 as an indicator for mitotic cells. As an example of application of this method to analysis of the mode of action of anti-tumor agents, the effects of podophyllotoxin and etoposide on the cell cycle were examined in human gastric cancer cells.

MATERIALS AND METHODS

Cell culture and mitotic arrest Human gastric cancer cell cultures, KATO-III and Nakajima, were maintained in RPMI 1640 medium (Nissui Pharmaco. Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) at 37°C under 5% CO_2. The cell line was passaged 1 to 2 days before each experiment to ensure logarithmic proliferation. Doubling times of KATO-III and Nakajima were 36 h and 30 h, respectively.

Drugs Etoposide and podophyllotoxin were provided by Nippon Kayaku Co. Ltd. (Tokyo). Each compound was dissolved in dimethyl sulfoxide at a concentration of 0.1 *M* and stored at −20°C. The final concentration of dimethyl sulfoxide was less than 1%, a concentration that had no effect on control cultures. Etoposide and podophyllotoxin were added to the culture medium at final concentrations of 1.0 μg/ml and 0.075 μg/ml, respectively. These concentrations corresponded to the IC_{50} of KATO-III. Samples were obtained every 6 h and cell kinetics were examined by flow cytometry.

Staining and flow cytometry Samples of approximately 1×10^6 cells were washed twice in phosphate-buffered saline (PBS) containing 0.5% Tween 20 and, after centrifugation, fixed with 70% cold ethanol for 30 min. After being washed twice, the cells were suspended in 1 ml of mouse anti-p105 supernatant (monoclonal antibody 780-3) or a nonspecific control for 1 h. Then, the cells were washed twice and incubated for 30 min with 0.33 ml of goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate (FITC) (Cappel

[3] To whom all correspondence should be addressed.

Laboratories, Cochranville, PA) diluted 1:20 with PBS. The cells were then incubated in 1 ml of PBS containing 150 units of ribonuclease A (Sigma Chemical Co., St. Louis, MO) per milliliter of PBS for 20 min at 37°C. After centrifugation, the cells were suspended in 1 ml of the PBS containing Tween 20 and 20 μg/ml propidium iodide (PI)(Sigma Chemical Co., St. Louis, MO) for 15 min. Flow cytometry was done with an EPICS C apparatus (Coulter Electronics, Inc., Hialeah, FL). Blue light (485 nm) from a single Inova 90 argon ion laser was used to make both FITC and PI fluoresce; the bivariate distribution of p105 (green fluorescence from FITC, 520 nm) versus DNA content (red fluorescence from PI, 580 nm) was measured. The data obtained were analyzed with a computer program, STATPAC (Coulter). The mean coefficient of variation of the G_0G_1 peak was $4.3 \pm 1.2\%$ (range, 3.5–5.4%). About 20,000 cells of each sample were measured.

To examine the correlation between the mitotic index measured by microphotometry and the M phase fraction calculated by flow cytometry, 0.2 μg/ml Colcemid (demecolcine, Sigma D7385, 1 mg/ml in 95% ethanol) was added before harvesting. A portion was taken hourly and divided into two samples. One sample was used to prepare a hematoxylin/eosin-stained smear to calculate the mitotic index by microphotometry. Another sample was processed for flow cytometry to calculate the M phase fraction based on the p105/DNA scattergrams.

RESULTS

Figure 1 shows DNA histograms and p105/DNA scattergrams obtained every 6 h after harvesting in human gastric cancer cells, KATO-III with etoposide. The M phase cells are enclosed by a rectangle in the control p105/DNA scattergram. Mitotic cells existed in the upper part of the G_2 phase and were separated distictly from G_2 phase cells in the p105/DNA scattergrams, because p105 was expressed to a large amount in mitotic cells. At 12 h and 24 h, an accumulation of G_2M phase cells was observed in DNA histograms, but M phase cells were not observed in the p105/DNA scattergrams. The results indicated that etoposide caused a G_2 block. Figure 2 shows the effects of etoposide in another gastric cancer cell line, Nakajima. While an accumulation of S and G_2M phase cells were observed at 12 h and 24 h in DNA histograms, M phase cells were not observed in p105/DNA scattergrams, as in KATO-III. Etoposide caused a delay of S phase transit and arrest of cells in G_2 in Nakajima.

Podophyllotoxin seemed to have the same action, an accumulation of G_2M phase cells, in both cell lines in the DNA histograms (Figs. 3 and 4). However, these accumulations of G_2M phase cells were found to be an

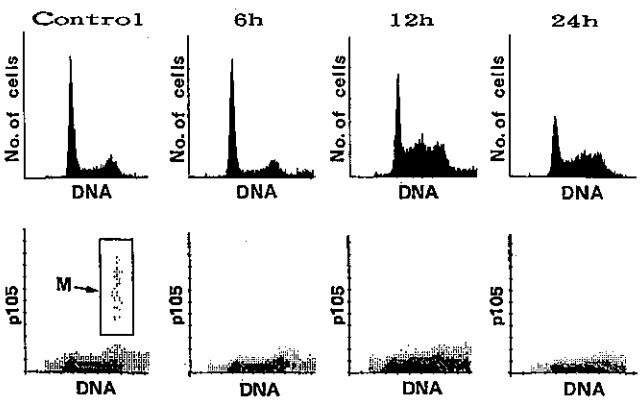

Fig. 2. Effects of etoposide on the cell cycle in human gastric cancer, Nakajima.

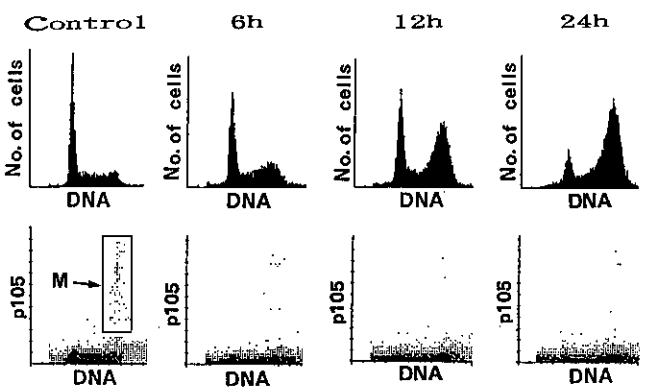

Fig. 1. Effects of etoposide on the cell cycle in human gastric cancer, KATO-III. Mitotic cells are enclosed by the rectangle in the control p105/DNA scattergram.

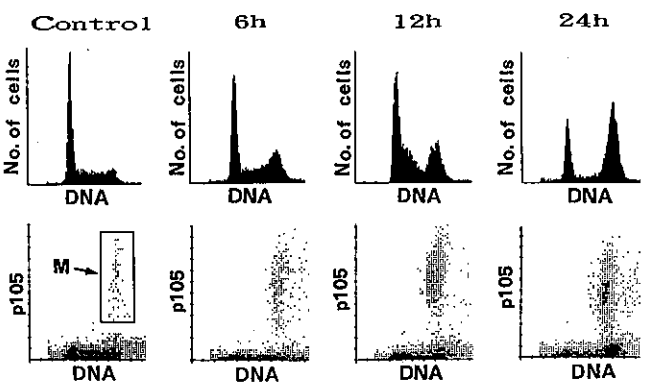

Fig. 3. Effects of podophyllotoxin on the cell cycle in human gastric cancer, KATO-III.

accumulation of not G_2 phase cells but M phase cells in the p105/DNA scattergrams. The degree of M phase block was different in each cell line. In the effects of podophyllotoxin on the cell cycle, G_2 block was dominant in KATO-III (Fig. 3), but M phase block was dominant in Nakajima (Fig. 4).

Figure 5 shows the correlation between mitotic indices measured by microphotometry and M phase fraction measured by flow cytometry. A high correlation ($r=0.92$, $P<0.001$) was observed between these factors.

The results of the cell cycle analyses of etoposide and podophyllotoxin in human gastric cancer cells are shown in Table I. The data may be summarized as follows: etoposide caused a G_2 block in KATO-III and retarded S phase transit and G_2 block in Nakajima. Podophyllotoxin caused a G_2 and M phase block in KATO-III and predominantly M phase block in Nakajima.

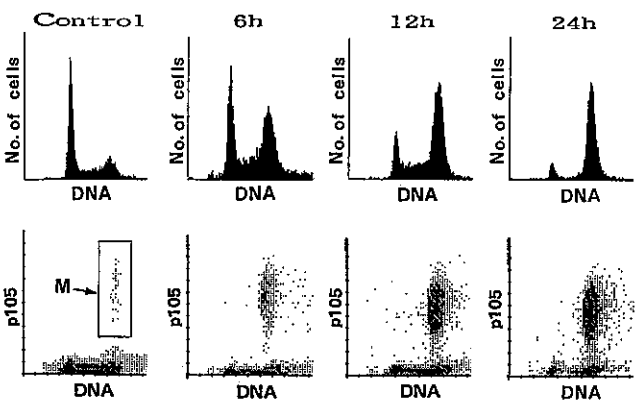

Fig. 4. Effects of podophyllotoxin on the cell cycle in human gastric cancer, Nakajima.

DISCUSSION

Several flow cytometric techniques have been used to discriminate mitotic cells from interphase cells. However, certain pretreatments of the cells or cell nuclei that induce or enhance differences between mitotic cells and G_2 phase cells were needed for these methods, for example, heating,[1] denaturation of DNA,[6] and BrdUrd incor-

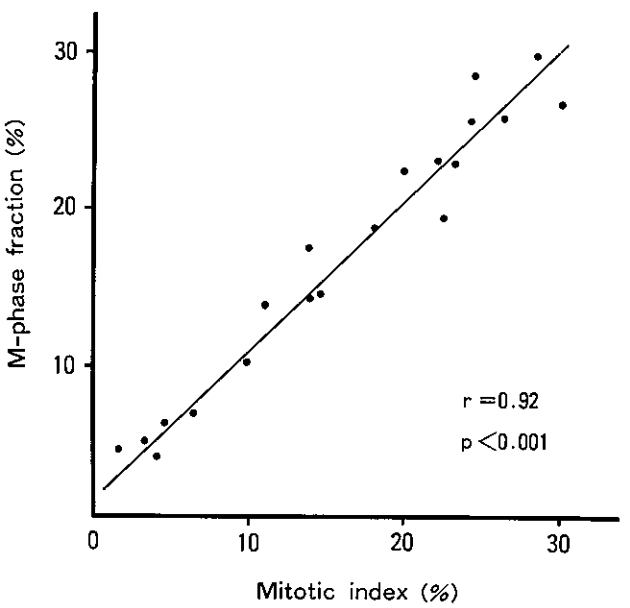

Fig. 5. Correlation between mitotic index measured by microphotometry and M-phase fraction measured by flow cytometry based on the p105/DNA scattergram.

Table I. Effects of Etoposide and Podophyllotoxin on the Cell Cycle in Human Gastric Cancer Cells

	Etoposide (1.0 μg/ml)				Podophyllotoxin (0.075 μg/ml)			
	Control	6 h	12 h	24 h	Control	6 h	12 h	24 h
KATO-III								
G_0G_1	46.6[a)]	36.8	28.3	13.7	46.6	40.2	33.9	22.6
S	24.7	35.3	15.2	21.7	24.7	19.0	19.5	17.0
G_2	27.6	28.0	56.1	64.5	27.6	38.4	41.2	51.1
M	1.1	0	0	0	1.1	2.4	5.4	9.3
Nakajima								
G_0G_1	41.7	41.3	18.5	9.2	41.7	34.7	15.0	10.9
S	28.1	28.6	50.4	50.8	28.1	24.5	24.5	16.4
G_2	29.2	30.2	31.1	40.4	30.2	22.6	18.9	1.4
M	1.0	0	0	0	1.0	18.2	41.6	71.3

a) Values are given as percentages.

poration.[2] We report here a method to distinguish mitotic cells from interphase cells by using anti-p105 monoclonal antibody. As described previously, p105 was expressed at 5–10-fold greater levels in mitotic cytoplasm and we used its greater expression in mitotic cells as an indicator of mitosis. p105 exists as an interchromatin granule in the nucleus and is thought to have a regulatory function in cell cycle progression. The staining method for p105 is quite simple and needs no pretreatment of cells. Furthermore, p105 could be stained in samples treated with several fixatives: paraformaldehyde, ethanol, formalin, and in even paraffin-embedded tissues.[10–12] p105 was not expressed in G_0 phase cells,[7] so that it was expected that we could distinguish G_0 phase cells from G_1 phase cells.

It was reported that etoposide, a semisynthetic derivative of podophyllotoxin with a potent inhibitory activity against eukaryotic DNA topoisomerase II, acts at a premitotic stage of the cell cycle to arrest cells in G_2 phase at low concentration and causes a retardation of S phase transit with continuous treatment or at a high concentration of etoposide.[13–15] On the other hand, podophyllotoxin prevents the formation of microtubules and additionally has a mitosis-arresting effect on the cell cycle.[16, 17] In our experiments, etoposide caused a G_2 block in KATO-III and retarded S phase transit and G_2 block in Nakajima, and podophyllotoxin caused a G_2 and M phase block in KATO-III and potential M phase block in Nakajima. These effects correspond with those previously reported.[14, 16] These different responses to the drugs, especially to podophyllotoxin, are due to the relative differences of the cytocidal drug concentrations. Nakajima is more sensitive to podophyllotoxin than KATO-III; the concentration (0.075 μg/ml) of podophyllotoxin required for 50% inhibition of the cell growth in KATO-III corresponds to that for 70% inhibition of the cell growth in Nakajima.

In conclusion, M phase cells were clearly separated from G_2 phase cells by using anti-p105 monoclonal antibody and flow cytometry. This method enabled us to perform reliable cell kinetic analysis easily and rapidly. The Colcemid block experiment confirmed this reliability and usefulness. Thus, multiparameter flow cytomety using anti-p105 monoclonal antibody should be useful for analysis of the modes of action of anti-tumor agents, especially for M phase analysis.

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