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In vitro combination effect of 5-fluorouracil and cisplatin on the proliferation, morphology and expression of Ki-67 antigen in human gastric cancer cells

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The combination effect of 5-fluorouracil (5-FU) and cisplatin was examined in terms of the proliferation, morphology and expression of Ki-67 antigen, and propidium iodide (PI) staining using four cultured human gastric cancer cell lines, and we assessed how such activity was affected by the exposure time and the timing of treatment. MKN-1, MKN-28, MKN-45 and MKN-74 cells were exposed to various concentrations of 5-FU for 72 h and cisplatin for 8 or 72 h. At IC_{50}, MKN-28 cells were more sensitive to 5-FU than other cell lines, whereas MKN-1 and MKN-45 cells were more sensitive to cisplatin than other cell lines. The cell growth-inhibitory activity of cisplatin was found to be 'area under the curve' dependent. When 5-FU and cisplatin were combined simultaneously, the combination effect was higher than that of 5-FU or cisplatin alone. On the other hand, when cisplatin was applied before 5-FU, there was no potentiation of the cell growth-inhibitory activity by combination treatment. In 5-FU-treated MKN-74 cells, the size, morphology and PI staining of nuclei were almost the same as those of the untreated cells, and the expression of Ki-67 antigen was less than that of the untreated cells. In cisplatin-treated MKN-74 cells, the size of cells and nuclei was larger than that of the untreated cells and fragmentation of nuclei was observed. The expression of Ki-67 antigen was less than the untreated cells but more than that of 5-FU-treated cells. In the cells treated with 5-FU and cisplatin in combination, the above changes were an intermediate of those of 5-FU-treated cells and cisplatin-treated cells. In conclusion, the cell growth-inhibitory activity of 5-FU and cisplatin against gastric cancer cells was potentiated by the combined treatment with 5-FU and cisplatin simultaneously, but not with cisplatin followed by 5-FU.

Key words: 5-Fluorouracil, cisplatin, combination effect, gastric cancer.

Introduction

5-Fluorouracil (5-FU) is one of the most widely used anti-cancer drugs in the treatment of gastrointestinal cancers and continuous infusion often gives better results. However, the response rate of 5-FU monotherapy is only about 10–30%[1–4] and its efficacy is not satisfactory. Therefore, various regimens of combination chemotherapy have been devised to enhance the clinical response of 5-FU in gastric cancers clinically[5–13] and cisplatin has been demonstrated to be one of key drugs for such purposes.[8,10–13]

The experimental basis for combination chemotherapy with 5-FU and cisplatin was first reported by Scanlon in 1986.[14] Cisplatin was demonstrated to inhibit the transport of methionine from the culture medium into human ovarian carcinoma A2780 cells and, as a result, intracellular methionine synthesis increased. This led to a rise in the intracellular concentration of reduced folic acid and a 2.5-fold increase in thymidylate synthase inhibition. Thus the concept of biochemical modulation was applied for this combination chemotherapy and thereafter several other reports appeared.[15–21] However, various treatment regimens have been used and the optimal treatment schedule is controversial.

This study was designed to evaluate the combination effect of 5-FU and cisplatin against four human gastric cancer cell lines in terms of cell growth-inhibitory activity, cell morphology, DNA staining with propidium iodide and immunostaining of Ki-67 antigens, as compared with 5-FU or cisplatin alone at various exposure times, and to assess how such activity was affected by the exposure time to cisplatin and the timing of treatment.

Materials and methods

Chemicals

5-FU was provided by Kyowa Hakko Kogyo (Tokyo,

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Japan) and cisplatin by Nippon Kayaku (Tokyo, Japan). PI was purchased from Sigma (St Louis, MO).

Cell culture

Four types of cultured human gastric cancer cell lines, MKN-1, MKN-28, MKN-45 and MKN-74, were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan), and used in the experiments. The number of cells was counted using a microscope, and the cells were cultured in RPMI 1640 culture media (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD), 100 units penicillin and 100 μg/ml streptomycin (Gibco BRL) at 37°C in a CO_2 incubator under the following conditions: humidity 100%, CO_2 concentration 5% and air 95%.

Determination of cell proliferation

The cell lines described above were cultured in 96-well microplates (Sumitomo Bakelite, Tokyo, Japan) for 4 days at 37°C and 5% CO_2, and the optimal number of cells which confers logarithmic growth between day 0 and day 4 was determined; MKN-1, 5000 cells/well; MKN-28, 1000 cells/well; MKN-45, 5000 cells/well; and MKN-74, 4000 cells/well. The cells at the above numbers were applied to 96-well microplates on day 0, and exposed to various concentrations of 5-FU for 72 h and cisplatin for 8 or 72 h from day 1. On day 4, MTT assay was performed by the following procedure.[22] First, 10 μl of MTT solution (MTT Kit; Chemicon, Temecula, CA) was added to each well and the plates were further cultured at 37°C in 5% CO_2 for 4 h. Then 100 μl/well of lysis solution (isopropanol/0.04 N HCl) was added to each well and the plates were cultured overnight. After complete lysis of all reaction products, the absorbance was measured at a wavelength of 550 nm with a microplate reader.

The concentrations of 5-FU and cisplatin that were required for 50 or 80% growth inhibition of each cell line were determined by measuring the absorbance.

Morphological change and fluorescence staining of cells

MKN-74 cells (5×10^4 cells/chamber) were cultured in chamber slides (Nunc, Roskilde, Denmark) on day 0, and exposed to IC_{50} of 5-FU for 72 h and cisplatin for 8 or 72 h from day 1. On day 4, immunostaining of Ki-67 antigens and DNA staining with PI were performed by the following procedure with a minor modification of previous methods.[23–25] The cells were washed with cold PBS(−), fixed with 80% acetone solution at −20°C for 10 min, washed with cold PBS(−), blocked with 3% goat serum (Cedarlane Laboratories, Hornby, Ontario, Canada) at room temperature for 10 min and washed again with PBS(−). The cells were treated with 800 μl/chamber of mouse anti-human Ki-67 monoclonal antibody (Zymed Lab, South San Francisco, CA) at 4°C overnight, washed with cold PBS(−), treated with 50-fold diluted goat FITC-labeled anti-mouse IgG polyclonal antibody (Caltag, South San Francisco, CA) at room temperature for 30 min in the dark, washed with cold PBS(−), treated with 2 ml of 0.1% RNase (Sigma) and washed with cold PBS(−).

Then the cells were treated with 1 ml of PI solution (1 μg/ml) at 4°C for 20 min in the dark and embedded in Antifade (Sigma). As the control, cells were treated with 0.5% BSA (Sigma) instead of mouse anti-human Ki-67 monoclonal antibody. The microscopic observation of stained cells was performed using a Nikon fluorescence microscope.

Statistical analysis

The statistical significance was determined by Student's *t*-test.

Results

Growth-inhibitory activity of 5-FU and cisplatin against cultured human gastric cancer cell lines

The growth-inhibitory activity of 5-FU and cisplatin against four human gastric cancer cell lines was examined by MTT assay at various exposure times (Table 1). From the kinetic analysis of the cell-killing effect of 5-FU and cisplatin, 5-FU was shown to be a time-dependent drug, indicating that a longer exposure time was preferable, while cisplatin was shown to be an AUC-dependent drug, indicating that its activity was independent of the exposure time.[26,27] Therefore the exposure time of 5-FU was fixed to 72 h and that of cisplatin was compared between 8 and 72 h. At IC_{50}, MKN-28 cells were more sensitive to 5-FU than other cell lines, whereas MKN-1 and MKN-45 cells were more sensitive to cisplatin than other cell lines. As for the exposure time of cisplatin, the IC_{50} values at 72 h exposure were about three to nine times lower than those at 8 h exposure in all cell lines, indicating that the antitumor effect of cisplatin was AUC

Table 1. Growth-inhibitory activity of 5-FU and cisplatin against cultured human gastric cancer cell lines

Cell lines[a]	Exposure time					
	5-FU		Cisplatin			
	72 h		8 h		72 h	
	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}
MKN-1	0.63	47	2.0	9.2	0.58	2.9
MKN-28	0.19	2.9	8.0	25	0.92	4.5
MKN-45	0.32	3.0	2.0	6.7	0.50	2.6
MKN-74	0.34	20	11	35	1.4	8.0

[a]The cells were cultured on day 0 and treated with the drugs on day 1 for the indicated time. On day 4, the cell number was measured by MTT assay, and IC_{50} and IC_{80} values (μg/ml) were determined.

dependent, since the theoretical IC_{50} ratio between 8 and 72 h exposure was nine in the case of an AUC-dependent drug. When the ratios of IC_{50} and IC_{80} were compared in each cell line, those of cisplatin were less than those of 5-FU, suggesting that the dose-response curve of cell growth-inhibitory activity of cisplatin was steeper than that of 5-FU.

Combination effect of 5-FU and cisplatin

Using four human gastric cancer cell lines, the combination effect of 5-FU and cisplatin was examined. The schedules of combination were as follows: (i) 5-FU 72 h and cisplatin 72 h (simultaneously), (ii) 5-FU 72 h and cisplatin 8 h (simultaneously), and (iii) cisplatin 8 h followed by 5-FU 64 h. These combination effects were compared with the effects of 5-FU or cisplatin alone, respectively, at the IC_{50} determined in Table 1 (Figures 1–4). When 5-FU and cisplatin were combined for 72 h simultaneously, the combination effect was higher than that of 5-FU or cisplatin alone against all four cell lines and these effects were statistically significant. When 5-FU and cisplatin were combined at 64–72 and 8 h exposure, respectively, the combination effect was observed differently by the treatment schedule. Simultaneous treatment gave a statistically significant potentiation of the cell growth-inhibitory activity of each drug in three cell lines, except for MKN-1 cells. On the other hand, when cisplatin was applied before 5-FU, there was no potentiation of the cell growth-inhibitory activity by combination treatment.

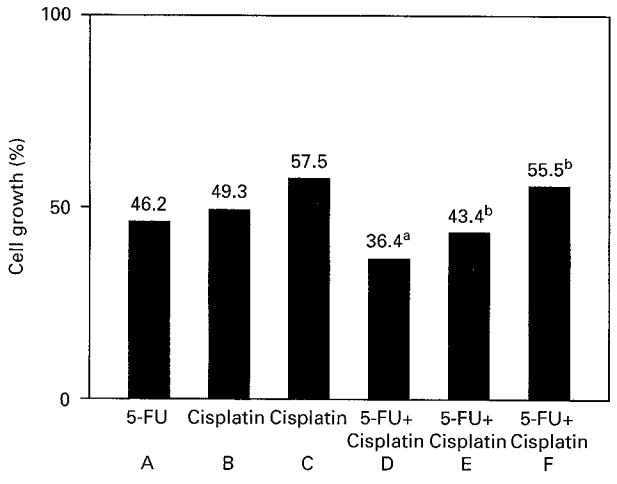

Figure 1. Combination effect of 5-FU and cisplatin against MKN-1 cells. The cells were cultured on day 0 and treated with 5-FU or cisplatin at IC_{50} for each exposure time from day 1 as follows; 5-FU for 72 h (A), cisplatin for 72 h (B), cisplatin for 8 h (C), 5-FU and cisplatin simultaneously for 72 h (D), 5-FU for 72 h and cisplatin for 8 h (E), or cisplatin for 8 h followed by 5-FU for 64 h (F). On day 4, the cell number was measured by MTT assay and the cell growth (%) was determined. [a]$p<0.05$ by Student's *t*-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (B), respectively. [b]Not significant by Student's *t*-test as compared with the 5-FU-treated group (A) and cisplatin-treated group (C).

Morphological change, the expression of Ki-67 antigens and PI staining

Using MKN-74 cells, the combination effect of 5-FU and cisplatin was examined in terms of the cell morphology, the expression of Ki-67 antigen and PI staining (Figure 5). 5-FU and cisplatin were combined simultaneously at 72 and 8 h exposure, respectively, since this treatment schedule gave a statistically significant combination effect in three cell lines including MKN-74 cells as shown in Figures 2–4. In the 0.5% BSA-treated control cells, nuclei were stained red by PI, but evidence of staining of Ki-67 antigen was not observed (Figure 5a). In the untreated cells, nuclei

were stained red by PI and some particles in a nucleus were stained yellow as a mixture of the red color of PI and the green color of FITC showing the expression of Ki-67 antigen (Figure 5b). Although the cytoplasm was stained green weakly, such color was also observed in the 0.5% BSA-treated control cells (Figure 5a), suggesting that this was a non-specific staining. In 5-FU-treated cells, the size, morphology and PI staining of nuclei (Figure 5c) were almost the same with those of the untreated cells (Figure 5b). The expression of Ki-67 antigens, which was found as particles, was less than that of the untreated cells. In cisplatin-treated cells for 72 h (Figure 5d) or 8 h (Figure 5e), the size of cells and nuclei was larger than that of the untreated cells (Figure 5b), and fragmentation of nuclei was observed. The expression of Ki-67 antigens was less than that of the control cells but more than that of 5-FU-treated cells. In the cells treated with 5-FU and cisplatin in combination, the size of cells and nuclei, the fragmentation of nuclei, and the expression of Ki-67 antigens were intermediate of 5-FU-treated cells and cisplatin-treated cells (Figure 5f and g). These results indicate that the combination effect of 5-FU and cisplatin was also observed in terms of these analyses.

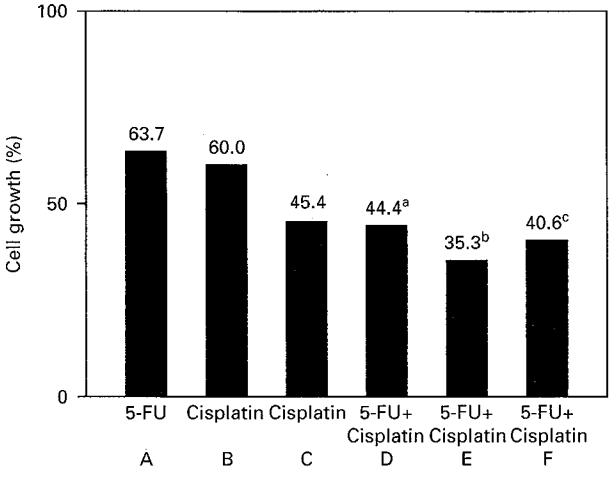

Figure 2. Combination effect of 5-FU and cisplatin against MKN-28 cells. The experimental protocol was the same with that of Figure 1. [a]$p<0.05$ by Student's *t*-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (B), respectively. [b]$p<0.05$ by Student's *t*-test as compared with 5-FU-treated group (A) and the cisplatin-treated group (C), respectively. [c]Not significant by Student's t-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (C).

Discussion

From the kinetic analysis of the cell-killing effect of 5-FU and cisplatin, 5-FU was reported to be a time-dependent drug, indicating that the longer exposure time is preferable, while cisplatin was reported to be an AUC-dependent drug, indicating that its activity is

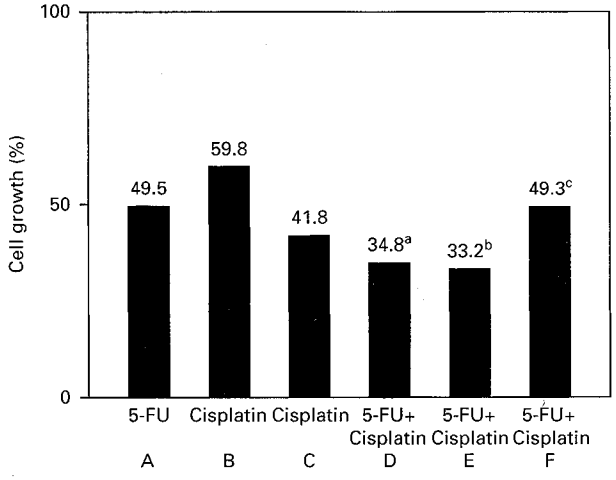

Figure 3. Combination effect of 5-FU and cisplatin against MKN-45 cells. The experimental protocol was the same with that of Figure 1. [a]$p<0.05$ by Student's *t*-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (B), respectively. [b]$p<0.05$ by Student's *t*-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (C), respectively. [c]Not significant by Student's *t*-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (C).

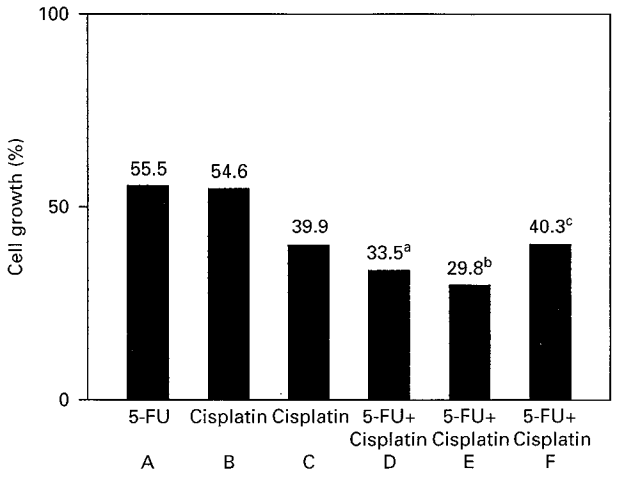

Figure 4. Combination effect of 5-FU and cisplatin against MKN-74 cells. The experimental protocol was the same with that of Figure 1. [a]$p<0.05$ by Student's *t*-test as compared with the 5-FU-treated group (A) and cisplatin-treated group (B), respectively. [b]$p<0.05$ by Student's *t*-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (C), respectively. [c]Not significant by Student's *t*-test as compared with the 5-FU-treated group (A) and the cisplatin-treated group (C).

independent of the exposure time.[26,27] Therefore, in this experiment, the exposure time of 5-FU was fixed to 72 h and that of cisplatin was compared between 8 and 72 h (Table 1). The IC_{50} values of cisplatin at

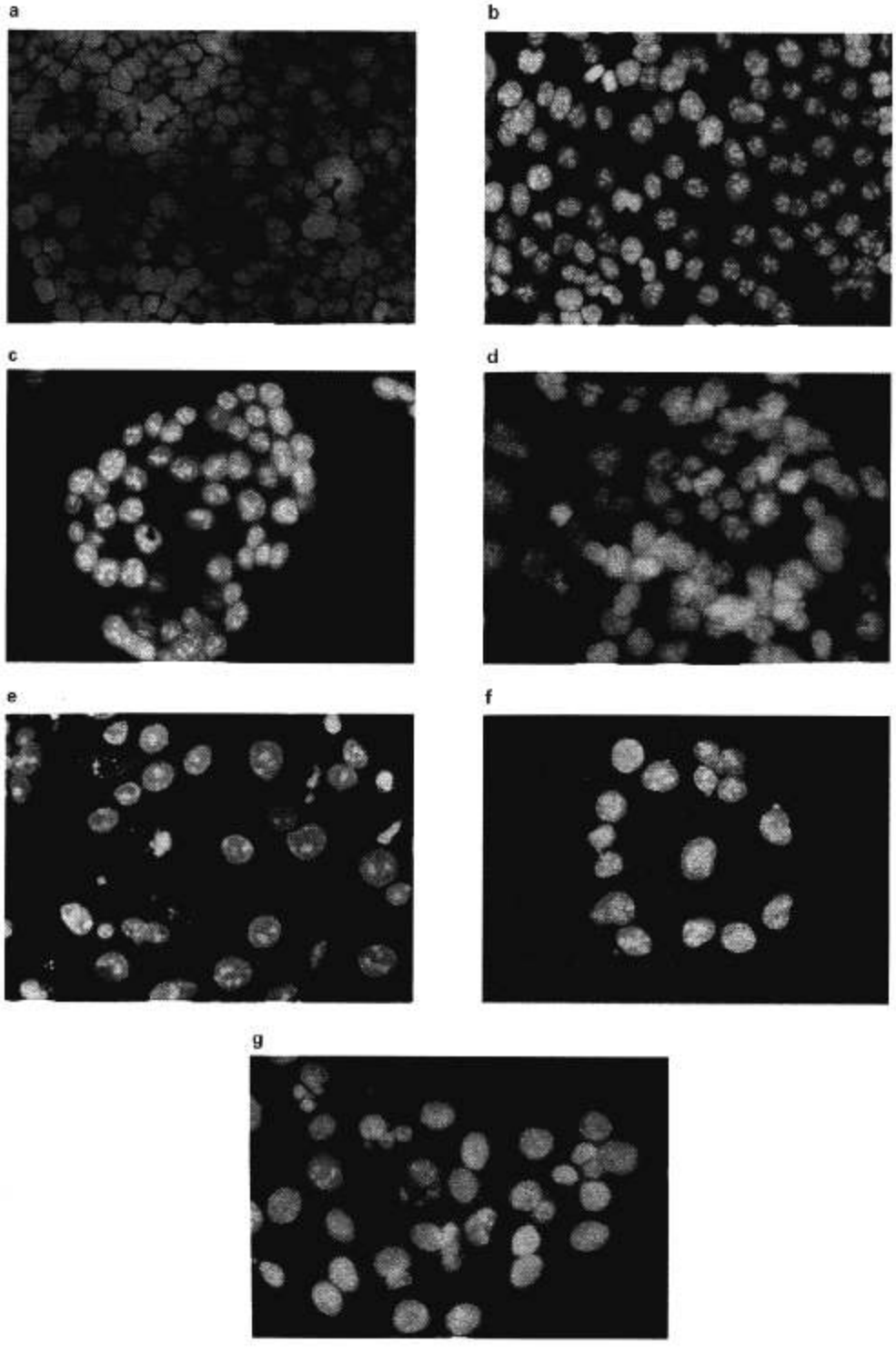

Figure 5. Double fluorescence staining of 5-FU- and cisplatin-treated cells by Ki-67 monoclonal antibody and PI. (a) 0.5% BSA control; (b) untreated; (c) 5-FU for 72 h; (d) cisplatin for 72 h; (e) cisplatin for 8 h; (f) 5-FU and cisplatin for 72 h; (g) 5-FU for 72 h and cisplatin for 8 h. Anti-Ki-67 antibody was treated in groups (b)–(g), and PI staining in groups (a)–(g). Magnification ×300.

72 h exposure were about three to nine times lower than those at 8 h exposure in all cell lines, indicating that the antitumor effect of cisplatin was AUC dependent, since the theoretical IC_{50} ratio between 8 and 72 h exposure was nine in the case of an AUC-dependent drug.

Various combination chemotherapy regimes have been devised to enhance the clinical response of 5-FU in gastric cancers clinically[5-13] and cisplatin has been demonstrated to be one of the key drugs for such purposes.[8,10-13] The experimental basis for this combination chemotherapy was firstly reported by Scanlon in 1986.[14] Cisplatin was demonstrated to inhibit the transport of methionine into human ovarian carcinoma A2780 cells and, as a result, intracellular methionine synthesis increased. This led to a rise in the intracellular concentration of reduced folic acid and a 2.5-fold increase in thymidylate synthase inhibition. Thus a regimen of cisplatin treatment followed by 5-FU treatment was proposed and was confirmed by some investigators.[15,17] However, other investigators reported that the regimen of 5-FU treatment followed by cisplatin treatment gave a better combination effect in terms of antitumor activity and side effects,[16,18-20] and a recent report[21] and our present results support the later regime. When 5-FU and cisplatin were combined simultaneously, the combination effect was higher than that of 5-FU or cisplatin alone (Figures 1-4). On the other hand, when cisplatin was applied before 5-FU, there was no potentiation of the cell growth-inhibitory activity. Although we could not examine the combination effect of 5-FU followed by cisplatin because the exposure time of 5-FU was fixed to 72 h, our results indicate that the regimen of cisplatin followed by 5-FU is not preferable. The mechanism of combination effect of 5-FU and cisplatin on the simultaneous schedule may be explained that the DNA-damaging activity of cisplatin is potentiated by treatment with 5-FU.[17,20,21]

Ki-67 antigen was reported to be expressed in the nuclei of proliferationg cells and to be a good indication of cycling cells.[23,24] 5-FU was reported to reduce the expression of Ki-67 antigens in both cultured cells and clinical specimens.[28,29] Our results using MKN-74 cells indicate that both 5-FU and cisplatin reduced the expression of Ki-67 antigen, which was recognized as yellow particles in the nuclei (Figure 5c-e). PI is a DNA intercalator, and penetrates only damaged cellular membranes and intercalates into DNA double strands.[25] In our experiments, since the cells were treated with 80% acetone solution, PI penetrated the cell membranes and intercalated DNA double strands, and subsequently the nuclei was stained red. In 5-FU-treated cells, the morphology of nuclei was almost the same as that of the untreated cells, whereas swelling and fragmentation of nuclei were observed in cisplatin-treated cells. In the cells treated with 5-FU and cisplatin in combination, the size of cells and nuclei, the fragmentation of nuclei, and the expression of Ki-67 antigens were intermediate of 5-FU-treated cells and cisplatin-treated cells (Figures 5f and g). These results indicate that the combination effect of 5-FU and cisplatin was also observed in terms of these analyses.

Conclusion

The cell growth-inhibitory activity of 5-FU and cisplatin against gastric cancer cells was potentiated by combined treatment with 5-FU and cisplatin simultaneously, but not with cisplatin followed by 5-FU.

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