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5-Fluorouracil + cisplatin + mitomycin C is a relatively most effective combination against xenograft lines of human colorectal cancer

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5-Fluorouracil (5-FU) has been an accepted effective against colorectal cancer, but combination regimens resulted in a lesser effect than 5-FU alone. The present study was designed to evaluate the efficiency of various combination chemotherapies, including 5-FU, on human colorectal cancer xenograft lines (CC-KK and RC-TK), which had been passed by transplantation in nude mice. Eight anticancer agents [5-FU, mitomycin C (MMC), adriamycin (ADR), 4′-epirubicin (EPIR), carboquone (CQ), cisplatin, carboplatin and etoposide (VP-16)] and their combinations were evaluated at 2–4 times the clinical dose. When the agents were administered singly, 5-FU, EPIR and CQ were effective against CC-KK, and 5-FU, MMC, EPIR, ADR, CQ and carboplatin were effective against RC-TK. Of the two-agent combinations including 5-FU, cisplatin + 5-FU was the most effective against both CC-KK and RC-TK. However, of the three-agent combinations, only cisplatin + 5-FU + MMC was more effective against both lines than cisplatin + 5-FU. These results suggest that cisplatin + 5-FU + MMC may be the most useful regimen against colorectal cancers in clinical application.

Key words: Chemotherapy, colorectal cancer, nude mice.

Introduction

Colorectal cancer is one of the most frequent causes of cancer death in developed countries. Most colorectal cancers are histologically well to moderately differentiated adenocarcinomas, and the prognosis of patients after a curative resection is comparatively better than patients with other digestive organ cancers, such as esophageal, pancreatic and hepatobiliary cancers. On the other hand, colorectal cancer is highly metastatic to the liver, and these metastatic lesions and the recurrent ones after surgery are highly resistant to various therapies, including chemotherapy, radiation, immunotherapy and hyperthermia. Although numerous clinical trials on new agents and combination regimens have been studied in order to improve the results of colorectal cancer chemotherapy, colorectal cancer still remains highly resistant to chemotherapy and only 5-fluorouracil (5-FU) has been accepted as an effective anticancer agent (ACA).[1–3] Accordingly, it is important to establish more effective combination regimens for colorectal cancer than 5-FU alone.

The present study was designed to evaluate the efficiency of various combination chemotherapies, including 5-FU, on human colorectal cancer xenograft lines (CC-KK and RC-TK) which had been passed by transplanting into nude mice.

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Materials and methods

Animals

Congenital athymic BALB/c nude mice (*nu+/nu+*), 6–8 weeks old, were purchased from CLEA Japan (Tokyo, Japan). They were bred and housed under specific pathogen-free conditions at the Kyoto University Laboratory Animal Center.

Human colorectal cancer xenograft lines (CC-KK and RC-TK)

The human tumor xenograft lines CC-KK and RC-TK were established from specimens resected in our department and were passed regularly by transplanting s.c. into the backs of nude mice. The origin of CC-KK is colon cancer and that of RC-TK is rectal cancer, and both lines are well to moderately differentiated adenocarcinomas, with a doubling time of 6.6 ± 0.5 and 10.0 ± 0.2 days, respectively.

Anticancer agents

The anticancer agents evaluated in this study and their dosages are summarized in Table 1. The original

Table 1. Dose of anticancer agents evaluated against CC-KK and RC-TK (in mg)

Agent	MCD[a] (/kg)	4 × MCD (/kg)	MCD (/m^2)
5-Fluorouracil (5-FU; Kyowa Hakko, Tokyo, Japan)	10.0	40.0	80.0
Mitomycin-C (MMC; Kyowa Hakko, Tokyo, Japan)	0.08	0.32	0.64
Adriamycin (ADR; Kyowa Hakko, Tokyo, Japan)	09.8	3.2	6.4
4'-Epirubicin (EPIR; Kyowa Hakko, Tokyo, Japan)	1.2	4.8	9.6
Carboquone (CQ; Sankyo, Tokyo, Japan)	0.16	0.64	1.28
Cisplatin (Bristol Myers, Tokyo, Japan)	0.9	3.6	7.2
Carboplatin (Bristol Myers, Tokyo, Japan)	9.0	36.0	72.0
Etoposide (VP-16; Britsol Myers, Tokyo, Japan)	3.0	12.0	24.0

[a] Maximal clinical dose.

carboplatin solution was used undiluted and VP-16 was diluted with 5% ethanol. All of the other agents were dissolved or diluted with distilled water. All agents were administered via an i.v. route at a dosage of 4 times their respective MCD/kg body weight; the MCDs were approved by the Japanese Ministry of Welfare and Health. In mice, these doses approximate only half of the MCD/m^2 body surface area.[4] These doses may have been maximal for nude mice in the three-agent combination regimens, because some combination regimens resulted in high mortalities in this study.

Protocols of drug administration (Figure 1)

After a cube of tumor tissue (3–4 mm, 15–30 mm^3) was transplanted s.c. into the backs of nude mice, its size was measured serially with calipers. To estimate the tumor volume (V), the following formula was used:[5] $V = L \times W^2 \times 1/2$, where L indicates the length and W indicates the width of the tumor.

When the tumor grew to 100–300 mm^3, which was usually 2–3 weeks after the transplantation, the drugs were administered (day 0). Tumor-bearing mice were randomized into two groups of four to six mice each: treated and control groups. Single-agent, two-agent, and three-agent combinations were then evaluated in three successive experiments. Each experiment was repeated twice and the results were calculated from the accumulated data (each group included eight to 12 mice in total).

In experiment 1, each single agent was administered at 4 × MCD weekly on days 0, 7 and 14. In experiment 2, 5-FU was administered on days 0 and 10 at 4 × MCD, and another agent was administered at 4 × the MCD on days 4 and 14. In experiment 3, 5-FU was given as the basic agent at half dose (i.e. 2 × MCD) on days 0, 4, 10 and 14 (total dose of 5-FU was the same as in experiment 2), and another two agents were administered alternatively at 4 × MCD on days 0, 4, 10 and 14. 5-FU was arbitrarily selected as the basic agent, because it is the most completely evaluated anticancer agent for colorectal cancer. Furthermore, in experiment 1 of this study, 5-FU was significantly effective against both cancer lines. The efficacy of each regimen was assessed on day 28 after the start of the treatment; the duration from the termination of the treatment to the day of the evaluation was 14 days. This drug-free duration is important in assessing the rebound growth phenomenon after chemotherapy. Mice in the control group were given 0.1 ml of saline. The efficacy and toxicities of each regimen were evaluated in terms of the percent inhibition (IR), the loss of body weight and the mortality, according to the following formulae. To estimate the relative tumor growth ratio (RTGR), the percent inhibition of the tumor growth ratio (IR) and the body weight ratio (BWR), the following formulae were used, respectively:

$$\text{RTGR} = V_n/V_0$$

$$\text{IR (\%)} = (1 \times \text{agent administered RTGR/control RTGR}) \times 100\%$$

$$\text{BWR} = \text{body weight on day } n\text{/body weight on day 0}$$

where V_0 indicates the tumor volume at the first administration (day 0) and V_n indicates the tumor volume at n days after the administration.

Statistics

All results were expressed as means ± SD and the p values were determined by Student's t-test using Medical Plan II computer software (Sankyo).

Results

The antitumor effects of single agents against CC-KK and RC-TK and their toxicities are summarized in

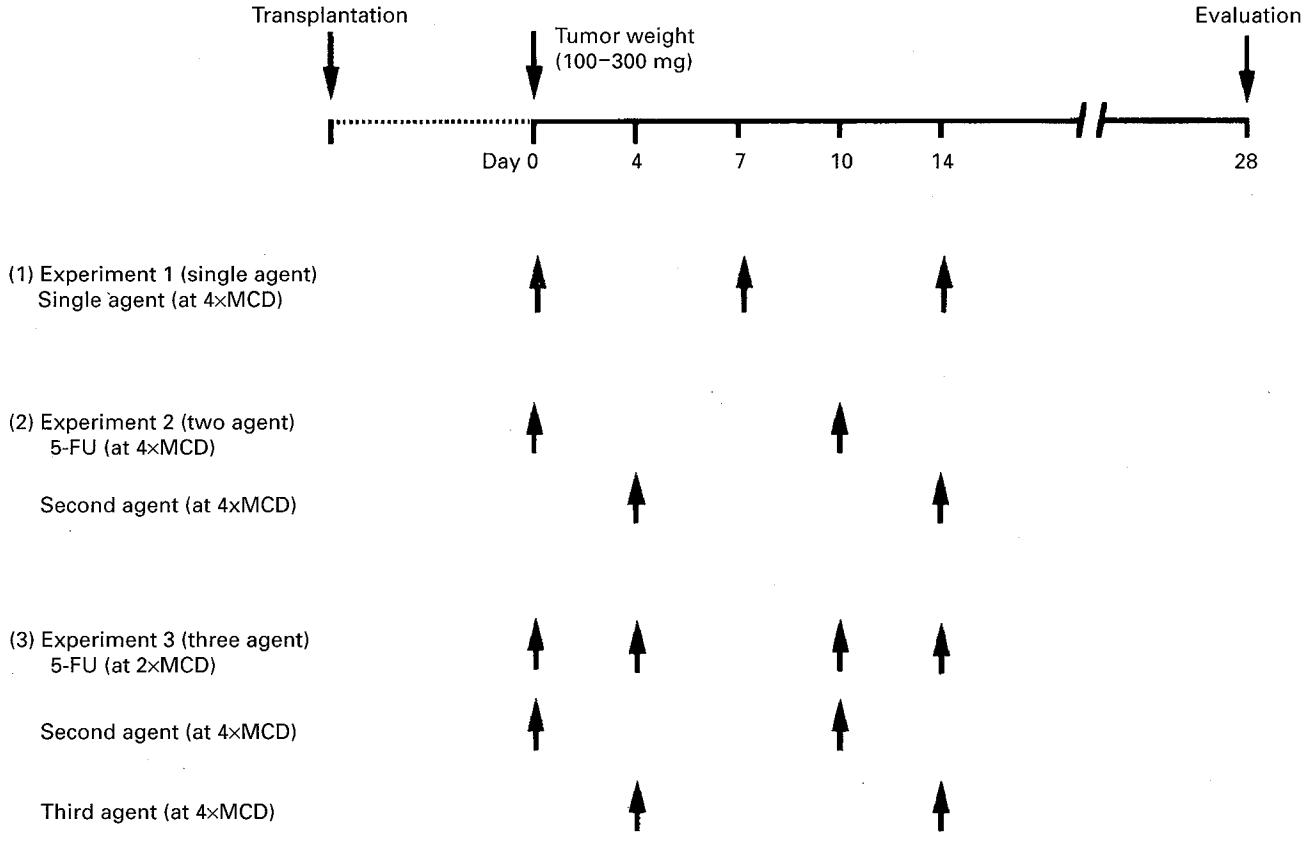

Figure 1. Protocol for the drug administration. Drugs were given when the tumor had grown to 100–300 mm^3, usually at 2–3 weeks after the transplantation. Tumor-bearing mice were randomized into groups of four to six mice each, i.e. the treated and control groups. Each experiment was repeated twice and the results were calculated from the accumulated data (each group included eight to 12 mice in total). In experiment 1, each single agent was administered at 4 × MCD (Table 1) weekly on days 0, 7 and 14. In experiment 2, 5-FU was administered on days 0 and 10 at 4 × MCD, and another agent was administered at 4 × MCD on days 4 and 14. In experiment 3, 5-FU was given as basic agent at half dose (i.e. 2 × MCD) on days 0, 4, 10 and 14 (total dose of 5-FU was the same as in experiment 2) and other two agents were alternatively administered at 4 × MCD on days 0, 4, 10 and 14. The efficacy of each regimen was assessed on day 28 after the start of treatment; the duration from the termination of the treatment to the day of evaluation was 14 days. The mice in the control group were given 1 ml of saline.

Table 2. Of the single agents, EPIR, CQ and 5-FU were significantly effective against CC-KK, with a mean IR of 70.9, 69.4 and 63.5%, respectively. In contrast, ADR, 5-FU, EPIR, CQ, MMC and carboplatin were significantly effective against RC-TK, with a mean IR of 63.3, 59.2, 57.7, 48.3 and 41.2%, respectively. No significant loss of body weight or mortality was noted in the groups receiving single agents. 5-FU, EPIR and CQ were effective against both lines.

Of the two-agent combinations (Table 3), 5-FU + cisplatin (FP) was the most effective regimen against CC-KK with an IR of 81.5% and showed a significantly higher inhibition than 5-FU alone. 5-FU + MMC (FM) or 5-FU + EPIR (FEpi) also showed higher inhibitions (IR, 74.3% and 74%, respectively) against CC-KK than 5-FU alone, but not significantly so. The combinations of 5-FU with ADR, CQ, carboplatin or VP-16 (FA, FQ, FCa or FE, respectively) did not augment the efficacy of 5-FU against CC-KK. RC-TK was resistant to the two-agent combinations. The FP regimen showed the highest inhibition (IR, 73.9%) against RC-TK, but it was not higher than 5-FU alone (IR, 71.4%). Furthermore, the other combinations shower a significantly lower inhibition against RC-TK than 5-FU alone. Of the seven combinations, the FA, FEpi and FE regimens resulted in a significant loss of body weight, and the FE regimen resulted in a 30% mortality.

Based on the results of the two-agent regimens, the three-agent combinations were designed to assess a combination of three agents from 5-FU, cisplatin, MMC or EPIR. These were the only four agents tested, because 5-FU plus MMC (FM), EPIR (FEpi) or cisplatin (FP) showed higher inhibitions against CC-KK than 5-FU alone, although FM and FEpi showed lower

Table 2. Tumor inhibitory effect and toxicities of single agents (experiment 1)

Protocol	Tumor inhibitory effect on				Toxicity	
	CC-KK (n=8–12)		RC-TK (n=8–12)		Body weight ratio (mean±SD)	Mortality (%)
	TGR[a] (mean±SD)	Mean IR[b] (%)	TGR (mean±SD)	Mean IR (%)		
Control	10.0±2.2	–	6.3±3.2	–	1.04±0.05	–
5-FU	3.6±1.6**	64.0	2.8±0.5*	55.6	0.99±0.01	0
MMC	13.1±8.1	−31.0	2.4±0.9*	61.9	0.98±0.03	0
ADR	7.8±5.0	22.0	2.2±0.5*	65.1	1.02±0.05	0
EPIR	2.4±1.6**	76.0	2.9±0.4*	54.0	0.95±0.02	0
CQ	2.5±1.6**	75.0	3.6±1.0	43.9	1.02±0.01	0
Cisplatin	13.9±7.6	−39.0	2.4±0.8*	61.9	1.00±0.02	0
Carboplatin	9.9±3.8	1.0	4.1±2.0	34.9	1.00±0.02	0
VP-16	13.3±5.5	−33.0	10.0±2.3	−58.7	1.02±0.02	0

All results were determined by the accumulated data from two independent experiments (n=8–12).
[a] TGR, tumor growth rate.
[b] IR, inhibition rate.
*p<0.05; **p<0.01; ***p<0.001 (versus control).

Table 3. Tumor inhibitory effect and toxicities of two-agent combinations (experiment 2)

Protocol	Tumor inhibitory effect on				Toxicity	
	CC-KK (n=8–12)		RC-TK (n=8–12)		Body weight ratio (mean±SD)	Mortality (%)
	TGR[a] (mean±SD)	Mean IR[b] (%)	TGR (mean±SD)	Mean IR (%)		
Control (saline)	10.0±3.5	–	7.5±2.5	–	1.05±0.04	–
5-FU alone	3.6±1.6	64.0	2.1±0.8	72.0	1.01±0.02	0
5-FU+MMC	2.6±1.9	74.0	5.8±3.0*	18.4	1.01±0.03	0
5-FU+ADR	3.7±2.9	63.0	3.10.7*	59.0	0.94±0.05##	0
5-FU+EPIR	2.6±2.5	74.0	4.0±2.4*	46.7	0.93±0.05##	0
5-FU+CQ	3.9±1.3	61.0	5.2±1.0**	30.7	1.01±0.02	0
5-FU+cisplatin	1.9±1/2*	81.0	2.0±0.5	73.3	1.00±0.002	0
5-FU+carboplatin	3.8±1.5	62.0	NT	–	1.03±0.06	0
5-FU+VP-16	3.5±2.2	65.0	NT	–	0.77±0.03###	0

All results were determined by the accumulated data from two independent experiments (n=8–12).
[a] TGR, tumor growth rate.
[b] IR, inhibition rate.
*p<0.05; **p<0.01 (versus 5-FU alone); ##p<0.01; ###p<0.001 (versus control).

inhibition against RC-TK than 5-FU alone. Table 4 summarizes the antitumor effects and toxicities of the three-agent combinations. FP+MMC (FPM) showed the highest inhibition against CC-KK with an IR of 88.8%, which was higher than FP (81.5%) in experiment 2. FPM also showed 75.4% IR against RC-TK, but this was almost the same as FP (73.9%) in experiment 2. FPEpi also showed a higher IR (88.3%) against CC-KK than FP, but showed a lower IR (63.8%) against RC-TK than FP. FPM did not cause body weight loss or death, but FPEpi resulted in a significant body weight loss and 33% mortality, and FMEpi caused significant loss of body weight.

Discussion

In the present study CC-KK was sensitive to 5-FU, EPIR and CQ, and RC-TK was sensitive to 5-FU, MMC, ADR, EPIR, CQ and carboplatin. This demonstrates the inherent heterogeneity and varaiable chemosensitivity of colorectal cancers. Only 5-FU, EPIR and CQ were effective against both lines. In the two-agent combinations, however, FP was only the regimen more effective than 5-FU alone. In the three-agent regimens, FPM was the most effective combination regimen against the two human colorectal cancer xenograft lines without serious side effects.

Table 4. Tumor inhibitory effect and toxicities of three-agent combination (experiment 3)

Protocol	Tumor inhibitory effect on				Toxicity	
	CC-KK (n=8–12)		RC-TK (n=8–12)		Body weight ratio (mean±SD)	Mortality (%)
	TGR[a] (mean±SD)	Mean IR[b] (%)	TGR (mean±SD)	Mean IR (%)		
Control (saline)	9.0±2.8	–	7.7±1.46	–	1.04±0.05	–
5-FU alone (experiment 2)		64.0		72.0	1.01±0.02	0
5-FU+cisplatin (experiment 2)		81.0		73.3	1.00±0.02	0
5-FU+cisplatin+EPIR	1.1±0.9**†	87.8	2.8±1.5**	63.6	0.90±0.04##	33
5-FU+cisplatin+MMC	1.0±0.3**†	88.9	1.9±0.4**	75.3	1.00±0.03	0
5-FU+MMC+EPIR	2.3±1.3**	74.4	NT	–	0.94±0.05##	0

All results were determined by the accumulated data from two independent experiments (n=8–12).
[a] TGR, tumor growth rate.
[b] IR, inhibition rate.
*p<0.05; **p<0.01 (versus control); †p<0.05 (versus 5-FU+MMC+EPIR); ##p<0.01 (versus control).

Of the various two-agent combinations only 5-FU + cisplatin (FP) regimen showed an augmented effect, compared to 5-FU alone, although cisplatin was ineffective against both lines, whereas 5-FU was effective against both lines. This is compatible with the observation of clinical chemotherapy. Since 5-FU is the most widely used agent for the chemotherapy against colorectal cancers and appears to be the only effective agent against colorectal cancer,[1–3] the effects of cisplatin against colorectal cancer were not clinically approved.[2] However, the FP regimen seems to be beneficial for colorectal cancer chemotherapy and it has been reported that the FP regimen resulted in a 20–35% response rate for colorectal cancer.[6–8] This augmenting effect of cisplatin on the antitumor activity of 5-FU can be explained as a biochemical modulation of 5-FU by cisplatin.[9,10] However, these reports also suggest that the FP regimen may not be useful, because the the addition of cisplatin to 5-FU did not improve the median survival, leading to the conclusion that the FP regimen should not be used in the routine management of patients with colorectal cancer.[2]

In the present study, three three-agent combinations were tested. The FMEpi regimen resulted in a lower inhibition rate than the FP regimen against CC-KK. The FPEpi regimen was effective against CC-KK, but also resulted in a lower inhibition rate against RC-TK than the FP regimen, with a significant loss of body weight and a 33% mortality. In contrast, the FPM regimen was effective against both lines without any serious side effects. Accordingly, FPM appears to be beneficial for colorectal cancer chemotherapy. Various three- or four-agent combination regimens have been tested against colorectal cancers; MOF (semustine + vincristine + 5-FU), MFO + streptozotocin and semustine + MMC + 5-FU are three representative regimens. However, these combination regimens did not show any greater likelihood of tumor regression than would have been anticipated with 5-FU alone.[11–13] In the present study, neither FPEpi nor FMEpi showed any superiority in comparison with the FP regimen or 5-FU alone (Table 4). In contrast, FPM showed a higher inhibition than 5-FU alone or the FP regimen, but only against CC-KK. There have been no reports on the clinical effects of this regimen for colorectal cancer, and this regimen may be worthy of evaluation in clinical trials.

The present study also demonstrated a very important issue in the design of combination regimens: the issue of drug interaction. The combination of effective agents did not always cause augmentation of effects, but instead antagonistic effects were sometimes observed. Furthermore, this effect was different between tumor lines. Our previous study also demonstrated these complex interactions between drugs in experiments using human digestive organ cancer xenograft lines, where the combination of effective agents did not cause augmentation of effects, but sometimes the combination of an effective agent with an ineffective agent works synergistically.[14] These results suggest that it is very difficult to combine agents by judging their effects when singly administered and may partly explain why combination chemotherapies are not always successful clinically. In the present study, a total of 12 two-agent regimens including 5-FU were assessed in two lines and only one regimen (FP) resulted in an augmented effect, whereas seven resulted in no augmentation and four resulted in decreased activity in comparison to 5-FU alone. This inefficiency in combining two agents was not due to increased side effects, because the mortality and body

weight of the mice were not affected by these combinations. Furthermore, it is notable that the FP regimen, which was the most effective two-agent combination, consists of an effective agent and an ineffective agent against both lines. There may be various factors responsible for this inefficiency in combining agents, including pharmacological antagonism, inadequate scheduling and dosing for the combinations. The most likely explanation is that the combined agents kill the same cell populations in the tumor. The agents tested in the present study retarded cell growth by inhibiting DNA synthesis in tumor cells: MMC and CQ through the alkylation of DNA,[15,16] cisplatin, carboplatin and MMC through DNA cross-linking,[17,18] ADR and EPIR through intercalation,[19] VP-16 through an inhibition of topoisomerase II,[20] and 5-FU by inhibiting thymidylate synthetase.[21] Similarities in the pharmacological actions of these agents may also be responsible for the ineffectiveness of their combinations.

It is time consuming, very expensive and a great disadvantage for patients to screen effective therapies from the numerous new agents and regimens available by clinical trials; therefore, although the screening system in nude mice is also expensive, we believe that this may be a very beneficial procedure and the only way to predict which regimen will be hold the best promise for clinical trials.

Conclusion

The present study demonstrates that FPM may be worthy of future clinical trials and may improve the results of chemotherapy against colorectal cancer. However, the present study also confirmed that 5-FU alone is still as effective as the various combination regimens, as was previously reported. The present study also demonstrated that xenograft lines in nude mice may provide a useful *in vivo* model for the design of combination regimens, which may have clinical applications.

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