Dual Antitumor Effects of 5-Fluorouracil on the Cell Cycle in Colorectal Carcinoma Cells: A Novel Target Mechanism Concept for Pharmacokinetic Modulating Chemotherapy

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ABSTRACT

5-Fluorouracil (5-FU) is one of the most widely used anticancer agents for advanced colorectal carcinoma, but its response rate is only 15%. The "pharmacokinetic modulating chemotherapy" (PMC) regimen that we have advocated has proved to be highly effective in treating colorectal carcinoma. PMC consists of a continuous i.v. infusion of 5-FU over 24 h for 1 day a week at 600 mg/m²/day, and an oral dose of uracil-tegafur (UFT), a 5-FU derivative, at 400 mg/day for 5–7 days per week, repeated every week for more than 6 months. Assays of 5-FU in 23 patients receiving this treatment showed serum concentrations ranging from 88 to 1323 ng/ml. We then analyzed the effects of clinically relevant concentrations of 5-FU found in colorectal cancer patients treated with the PMC regimen on the growth of three human colorectal adenocarcinoma cell lines, SW480 and COLO320DM (mutant p53) and HCT116 (wild-type p53). Exposure of these three cell lines to 5-FU resulted in growth inhibition in a dose-dependent manner. Exposure to 100 ng/ml of 5-FU in SW480 and COLO320DM caused G₁ arrest after 24 h and G₂ arrest after 72–144 h, and only a minority of the cell population showed apoptotic features, which indicated that most of the cells were killed through mitotic catastrophe, nonapoptotic cell death. On the contrary, exposure to 1000 ng/ml of 5-FU in SW480 and COLO320DM resulted in G₁-,S-phase arrest and the induction of apoptosis throughout the experimental period. Nucleolar cyclin B1 expression was markedly induced with exposure to 100 ng/ml of 5-FU in SW480 and COLO320DM; and expression of 14-3-3 protein, a cell cycle inhibitor in the G₂ phase, was induced in SW480. HCT116 responded to lower concentrations of 5-FU more rapidly: G₁ arrest was seen after 24–72 h of exposure to 10 ng/ml of 5-FU, and G₁ arrest was seen after 12–24 h of exposure to 100 ng/ml of 5-FU. These results show that 5-FU acts via two different pathways, depending on dose: (a) G₁-,S-phase cell cycle arrest and apoptosis at 1000 ng/ml in SW480 and COLO320DM, and 100 ng/ml in HCT116; and (b) G₂-M-phase cell cycle arrest and mitotic catastrophe at 100 ng/ml in SW480 and COLO320DM, and 10 ng/ml in HCT116. These results suggest that the efficacy of our PMC regimen is based on targeting at least two different phases of the cell cycle. In our clinical trial, we showed efficacy independent of p53 status, ascertainment by cell kinetic analysis in vitro, which may lead to a novel concept of schedule-oriented biochemical modulation of this drug.

INTRODUCTION

Maximizing therapeutic response by increasing selectivity is a major goal in the development of anticancer therapy. 5-FU is still one of the most widely used agents in the first-line therapy of colorectal carcinoma, although various regimens have been developed over the past 30 years (1–4). The limited therapeutic response of only 15% in advanced colorectal carcinoma has encouraged many modifications of the schedule of 5-FU administration, using various modulators including cisplatin, N-(phosphonomethyl)-L-aspartic acid (PALA), thymidine, methotrexate, and LV (1–4). Currently, 5-FU plus LV is widely used to treat advanced colorectal carcinoma. However, its efficacy is still controversial, because this combination has not yet been shown to contribute significantly to an improvement in overall survival, despite advantages over 5-FU alone, in terms of objective responses (1, 5–9). Additionally, severe and intolerable side effects have been observed using this therapy limiting its application (10–12).

We designed a regimen of PMC, involving continuous i.v. infusion of 5-FU for 24 h per week and oral administration of UFT (Taiho Pharmaceutical Co., Tokyo, Japan) twice a day for 5–7 days per week (13, 14), based on experiments using rat models by Fujii et al. (15). UFT is a combination of tegafur, a produg of 5-FU, and uracil at a molar ratio of 1:4 (15). DPD, a key enzyme in the degradation of 5-FU into therapeutically inactive metabolites, catalyzes the reduction of 60–90% of administered 5-FU, and its catalytic activity correlates with the rate of 5-FU clearance (16). Uracil inhibits hepatic DPD and thus enhances the plasma 5-FU level and the antitumor activity of 5-FU (17). Our PMC regimen has drastically improved the prognosis of patients with advanced colorectal carcinoma over the past 10 years (18, 19). Mutations of the p53 gene have been found in ~50% of colorectal carcinomas and are associated with lymphatic dissemination and a poorer prognosis (17, 18). We found, in comparison with patients bearing tumors with p53 mutation in non-PMC, those in PMC showed a lower local recurrence rate (0% in PMC versus 28.6% in non-PMC), a lower distant recurrence rate (7.1% in PMC versus 42.9% in non-PMC), and a higher 3-year survival rate (100% in PMC versus 64.3% in non-PMC; Ref. 13). Subsequently, PMC significantly improved the prognosis of unresectable colorectal carcinoma (median survival of 26.6 months with PMC versus 9.2 months with non-PMC; Ref. 14). Moreover, our regimen was well tolerated, with only 2 (3.6%) of 56 patients showing grade 3 or 4 toxicity and no deaths (14). Analyzing the relationship between the optimal plasma concentration range and the administered dose of 5-FU, we found that little antitumor activity was reported at 5-FU plasma level of 50 ng/ml for 6 consecutive days (15), and UFT administration (300 mg/m²/day) for 28 consecutive days did not lead to any partial or complete response (20). The plasma 5-FU concentration was reported to reach a maximum of 430 ng/ml after 2 h of UFT administration and 90 ng/ml of a stasis even after 6 h of it (21). On the other hand, a much higher dose (µg/ml scale, e.g., 50 µg/ml) of 5-FU, which would be difficult to reproduce clinically, has been used in in vitro experiments to show cytotoxic effects (22). Taken together, our successful trial suggested that the efficacy of 5-FU is dependent on the administration regimen, i.e., there may be two or more different cytotoxic mechanisms, with different thresholds, in a rather narrow therapeutic range.

Cell cycles are halted at the transition from G₁ to S-phase (G₁ checkpoint) or from G₂ to M-phase (G₂ checkpoint) after DNA replication.

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2 The abbreviations used are: 5-FU, 5-fluorouracil; PMC, pharmacokinetic modulating chemotherapy; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; UFT, uracil-tegafur; LV, leucovorin; DPD, dihydropyrimidine dehydrogenase; cdk, cyclin-dependent kinase; MAb, monoclonal antibody.

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damage. In the G₁ checkpoint, cyclin D₁ and E regulate cell progression and provide substrate specificity to their partners, the cdks, whereas the CIP/KIP family, including p21^{WAF1/CIP1} and p27^{KIP1}, or the INK4 family, including p16 and p19^{ARF}, act as inhibitors. In the G₁ checkpoint, initiation of mitosis in human cells requires the activation of M-phase-promoting factor, a complex of a cdk Cdc2 and a B-type cyclin (24–26). The activity of M-phase-promoting factor is regulated by dephosphorylation of Cdc2 and nuclear accumulation of cyclin B₁ protein (24–26). The nuclear translocation of cyclin B₁ is regulated by its phosphorylation and is known to mediate its biological activity (27), although how phosphorylation of cyclin B₁ leads to its nuclear translocation is still unclear. The Cdc2-cyclin B₁ complex, which accumulates in the cytoplasm during S and G₂ phases, translocates to the nucleus during the prophase of M phase, and thus, regulates cell cycle progression (28–32).

5-FU is generally believed to induce G₁-S-phase arrest, and its cytotoxic effects are attributed to apoptosis, via a p53-dependent pathway (33). A recent study has shown that p53 mutants are recessive to wild-type for growth arrest but were selectively negatively dominant for induction of apoptosis after DNA damage (34). However, the relationship between the status of p53 gene and cell cycle regulation under exposure to 5-FU remains unclear.

In this study, we examined the effects of various concentration of 5-FU on cell cycle regulation using three human colorectal carcinoma cell lines with or without mutations of the p53 gene, based on the plasma concentration profile of 5-FU during the PMC regimen, and also investigated which cell cycle alterations operated clinically in cancer tissue obtained from patients with colorectal carcinoma under PMC.

### MATERIALS AND METHODS

#### Patients and Treatment Protocol

Twenty-three patients who had undergone radical resection of colorectal carcinomas at the Second Department Surgery, Hyogo College of Medicine, volunteered to give blood samples during PMC and entered this preliminary study. All were given a complete explanation of the procedures involved, and informed consent was obtained. For continuous i.v. infusion in PMC, a catheter was inserted into a subclavian vein (6 Fr. Anthrone P-U catheter, Toray Medical Co., Tokyo, Japan), and was connected to a Cell site port (Toray Medical Co.). The port system was fixed s.c. PMC was started 2 weeks after surgery. The weekly protocol of PMC is as follows: continuous infusion of 5-FU (600 mg/m²) for 24 h on day 1, using a disposable pump, plus oral UFT at doses of 200 to 400 mg/body/day, twice a day, for at least 5 consecutive days on days 1–5 or 1–7. Patients in this series including PMC use for neoadjuvant chemotherapy underwent PMC preoperatively. Namely, the 5-FU infusion was performed 3 days before the surgery. Patients were recommended to continue on this regimen every week for more than 6 months. Colorectal cancer tissue specimens were obtained from the above-mentioned 23 patients, and were stored at −80°C for use in whole cellular protein extraction and flow cytometry.

#### Extraction and Measurement of the Plasma 5-FU Concentration

Blood samples were obtained at 5–11 time points serially from each patient during the first 24 h and were promptly centrifuged (3000 rpm, 10 min) at 4°C. Serum samples were stored at −20°C until use. The plasma 5-FU concentration was measured by high-performance liquid chromatography as described elsewhere (35).

#### Cell Culture and Cell Growth Study

Two colorectal adenocarcinoma cell lines with mutant p53, SW-480 and COLO320DM (36), were obtained from the Human Science Research Resource Bank (Tokyo, Japan) and a colorectal adenocarcinoma cell line with wild-type p53, HCT116 (36), was purchased from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with fetal bovine serum [10% (v/v), HyClone, Logan, UT], glutamine (2 mM), penicillin (100,000 units/liter), streptomycin (100 mg/liter), and gentamycin (40 mg/liter). Cell cultures were maintained at 37°C, in a humid atmosphere of 5% CO₂,95% air. For the cell growth study, 10<sup>4</sup> cells were plated per 60-mm dish and treated with 5-FU (Kyowa Hakko Kagyo Co., Ltd. Tokyo, Japan) of concentrations indicated in Fig. 1. The medium was replaced on day 3. Cells were counted using a hemacytometer on the days indicated.

#### Preparation of Whole Cellular, Nuclear, and Cytoplasmic Extracts

**Whole Cellular Extracts.** Frozen surgical specimens and cultured cells were homogenized in ice-cold lysis buffer: PBS (–) supplemented with NP40 [1% (v/v)], sodium deoxycholate [0.5% (w/v)], and SDS [0.1% (w/v)], with protease inhibitors, as described previously (37). The lysate was gently rotated (60 min, 4°C) and centrifuged (15000 rpm, 30 min, 4°C), and supernatants were collected as whole cellular proteins.

**Nuclear and Cytoplasmic Extracts.** Cultured cells were homogenized with a dounce-homogenizer, and then suspended in ice-cold hypotonic lysis buffer: HEPES (10 mM), MgCl₂ (1.5 mM), KCl (10 mM), and protease inhibitors (37). The lysate was centrifuged (4000 rpm, 15 min, 4°C), and this supernatant was used as cytoplasmic extract. The pelleted fractions from the first spin were then suspended in an equal volume of nuclear extract buffer [a half-volume of high-salt buffer; HEPES (20 mM), glycerol (25%), MgCl₂ (1.5 mM), KCl (1.2 M), and EDTA (0.2 mM)] after a half-volume of low-salt buffer; HEPES (20 mM), glycerol (25%), MgCl₂ (1.5 mM), KCl (0.02 M), and EDTA (0.2 mM)]. Proteinase inhibitors, gently rotated (60 min, 4°C) and centrifuged (15000 rpm, 30 min, 4°C), were then suspended in 10% SDS-PAGE gel and used as whole cellular proteins.

**Western Blotting.** Western blots were performed as described previously (37). Briefly, samples of proteins (30 μg per lane) were subjected to a 10–20% gradient SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) using a Multiphor II (Amersham Pharmacia Biotech). The blotted membrane was immersed in 5% (v/v) skim milk for blocking and then incubated with the primary antibodies overnight at 4°C. It was subsequently incubated with the secondary antibodies coupled to alkaline phosphatase (5 h, 4°C). After extensive washing, immunoreactive bands were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate. The primary antibodies used were a mouse antihuman MAb that recognizes both the wild-type and mutant p53 (UBI, Lake Placid, NY), rabbit polyclonal Abs against human p19<sup>ARF</sup> (Biosource International, Camarillo, CA), p21<sup>WAF1/CIP1</sup>, p27<sup>KIP1</sup>, cyclin A, cyclin D₁, cdk2, cdk4, cdk6, and Cdc25C, and a goat polyclonal antibody against human cyclin 14-3-3 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse MAbs against human cyclin E (PharMingen, San Diego, CA), Cdc2, and cyclin B₁ (Santa Cruz Biotechnology). Anti-rabbit or -mouse or -goat IgGs conjugated with alkaline phosphatase (Santa Cruz Biotechnology) were used as secondary antibodies.

**Flow Cytometry.** Floating and trypsinized adherent cells were collected, suspended in PBS (–), fixed with 70% (v/v) ethanol, and stained with propidium iodide (50 μg/ml). Then the DNA content was analyzed using a FACSscan (Becton Dickinson, Lincoln Park, NJ) with CELL Quest and Mod Fit LT 1.0 software (Verity Software House, Inc., Topsham, ME). Cell debris and fixation artifacts were gated out.

**TUNEL Staining.** Apoptotic cells were detected using the MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya, Japan), based on the dUTP-biotin TUNEL method, to visualize DNA strand breaks.
Morphological Measurement of Surgical Specimens

Surgical sections were selected from the area including the maximum diameter of each tumor along the longitudinal axis. The examined sections of each tumor were those median and bilateral sections at intervals of 5 mm. The proportion of tumor nest to background stroma was quantitatively measured using the image analyzing system (IBAS 20, Zeiss, Oberkochen, Germany).

Immunohistochemistry

Colorectal cancer tissue specimens were obtained from the 23 patients, processed using conventional procedures for paraffin embedding, cut into 4-μm sections, and mounted onto poly-l-lysine-coated slides. Sections were dewaxed in xylene, and rehydrated in descending alcohols, heated in a microwave oven for 5 min twice for antigen retrieval, blocked for endogenous peroxidase with 3% H2O2 (v/v) in methanol, and then blocked for nonspecific antibody binding with normal rabbit serum. They were incubated overnight at 4°C with a mouse MAb against human cyclin B1 (DAKO, CA) followed by a standard avidin-biotin-peroxidase complex method. The slides were developed with 3,3’-diaminobenzidine tetrahydrochloride solution containing 0.1% H2O2 and were lightly counterstained with hematoxylin. Normal mouse IgG was substituted for the primary antibody as a negative control.

Statistical Analysis

Statistical analysis of the maximum plasma concentration of 5-FU with or without grade 3 toxicity was calculated with the Student t test, and P < 0.05 was considered statistically significant.

RESULTS

Plasma Concentration of 5-FU during PMC. The plasma concentration of 5-FU during 24 h on day 1 of PMC ranged from 88 to 1323 ng/ml in 166 samples taken from 23 patients. We began taking samples for measurements of the 5-FU plasma concentration immediately at the start of the 5-FU infusion. There were two peaks in the 5-FU plasma concentration during the 24 h infusion, which followed the two times of UFT administration. The median plasma 5-FU concentration of the first peak at 3–4 h after the first oral intake of UFT was 267 ng/ml (25% and 75% quartile; 173 and 386 ng/ml, respectively), and gradually decreased to 203 ng/ml at 12 h. That of the second peak was 402 ng/ml (25% and 75% quartile; 246 and 501 ng/ml, respectively) at 15–19 h, indicating a maximum at 3–7 h after the second oral administration of UFT, and decreased to 135 ng/ml at 24 h. The second peak was higher than the first in all but two patients. In the three patients with grade 3 toxicity, the median value of the maximum plasma concentration of 5-FU was 956 ng/ml, significantly higher than in the 20 patients without such toxicity (956 ng/ml versus 376 ng/ml, P = 0.008). The PMC regimen maintained the 5-FU concentration above 200 ng/ml for 20 h. UFT alone showed a maximum plasma concentration of 5-FU at 58.7 ng/ml under a non-PMC regimen in our previous study (38).

Suppression of Cell Growth by 5-FU. The above results demonstrate the relevant clinical therapeutic range of 5-FU is from about 50 to 1000 ng/ml. We examined the effects of 5-FU at these concentrations on the growth of colorectal carcinoma cell lines, which contain mutant (SW480 and COLO320DM) and wild-type p53 (HCT116). Exposure to 100 and 1000 ng/ml of 5-FU suppressed the growth of SW480 by 92% and 96% at 144 h, respectively (Fig. 1A) and COLO320DM by 97% and 98% at 144 h, respectively. HCT116 is much more susceptible to 5-FU than SW480 and COLO320DM; after exposure to 100 and 100 ng/ml of 5-FU for 144 h, its growth was inhibited by 86% and 99%, respectively (Fig. 1B).

DNA Flow Cytometric Analysis and Apoptosis. On flow cytometry, SW480 cells showed 34.9% in G0/G1, 45.0% in S, and 20.1% in G2-M phase before exposure to 5-FU (Fig. 2A). After treatment with 100 ng/ml and 1000 ng/ml of 5-FU for 24 h, approximately 50% of cells were arrested in G1 phase. Following exposure to 100 ng/ml of 5-FU for 72 to 144 h, G2-M-phase accumulation (24.0% and 38.2% at 72 and 144 h, respectively) was seen, whereas cells exposed to 1000 ng/ml of 5-FU remained in the G1 phase (42.6% at 72 h). At 144 h, apoptotic cells, which appeared in a region to the left of the G1 peak, accounted for 24.2% at 100 ng/ml, and 36.3% at 1000 ng/ml. The percentage of apoptotic cells visualized by the TUNEL technique (Fig. 2C) by treatment with 100 ng/ml was also lower than that with exposure to 1000 ng/ml (6.9% at 100 ng/ml versus 14.4% at 1000 ng/ml at 144 h). Similar flow cytometric distribution and apoptotic features were observed in COLO320DM cells (data not shown). In HCT116 cells, G2-M-phase accumulation was seen after exposure to 10 ng/ml of 5-FU (26.8, 40.7, and 45.5% at 0, 24, and 72 h, respectively) and G2-G1 and S-phase accumulation was noted after exposure to 100 ng/ml of 5-FU (64.1 and 76.7% at 0 and 24 h, respectively) compared with cells before exposure to 5-FU (Fig. 2B).

Effect of 5-FU on Expression of Cell Cycle Regulatory Proteins. Expression of cell cycle regulatory factors in SW480 and COLO320DM cells treated with 100 and 1000 ng/ml, and HCT116 cells with 10 and 100 ng/ml of 5-FU was analyzed at different time
points. No significant change in expression pattern of cdk2, cdk4, cdk6, cyclins D1 and E (Fig. 3), p27kip1, p53, p15, p16, p19arf, Cdc25C, or cyclin A (data not shown) was observed in any cell line (data of COLO320DM not shown). However, expression of p21waf1/cip1 was markedly increased in a time-dependent manner in SW480 at 100 ng/ml (Fig. 3A), and in dose- and time-dependent manner in HCT116 with wild-type p53 (Figs. 3B and 4). Induction of 14-3-3σ, a key protein for G2-M arrest (39), was clearly observed in a dose-dependent manner (at 1000 ng/ml; 4.6- and 4.7-fold of the 0 ng/ml level in SW480 and HCT116, respectively; Fig. 4) and a time-dependent manner in SW480 and HCT116 (at 144 h with 100 ng/ml; 4.7- and 2.1-fold of the 0 h level in SW480 and HCT116, respectively; Fig. 3). No expression of p21waf1/cip1 nor of 14-3-3σ was found in COLO320DM before or during exposure to 5-FU (data not shown). These results indicate that induction of 14-3-3σ is not dependent on wild-type p53 in colorectal carcinoma cells, although its expression has been reported to be dependent on wild-type p53 (39).

We also analyzed the intracellular localization of cyclin B1 and Cdc2, because their nuclear accumulation has been reported to correlate with mitotic catastrophe (39). After 24 h exposure to 100 ng/ml of 5-FU, nuclear cyclin B1 levels increased significantly in all three cell lines (2.3-, 4.0-, and 1.8-fold of the 0 ng/ml level in SW480, COLO320DM, and HCT116, respectively; Figs. 3 and 4). Notably, increases were seen at 100, 500, and 1000 ng/ml concentrations in SW480, whereas no increase was observed at concentrations above 100 ng/ml in HCT116 cells (Fig. 4). In a time course analysis, induction of nuclear cyclin B1 was seen after 24 h (2.2- and 3.9-fold of the 0 h level at 24 h with 100 ng/ml of 5-FU in SW480 and COLO320DM,
respectively, Fig. 3A), whereas it was seen during the earlier phase in HCT116 (2.9-fold of the 0 h level at 24 h with 10 ng/ml of 5-FU in HCT116; Fig. 3B). Similarly, nuclear Cdc2 accumulation was observed at 100 ng/ml of 5-FU, and longer exposure (144 h) was needed to see a substantial increase in SW480 (Fig. 3A) and COLO320DM, whereas nuclear translocation of Cdc2 was clear at 72 h exposure in HCT116 (Fig. 3B).

**DISCUSSION**

It has been 10 years since we started using the PMC regimen for the treatment of advanced colorectal carcinoma. We originally devised it because the Japanese National Health Insurance scheme did not cover the use of 5-FU with LV for colorectal carcinoma. There have been two different approaches to biochemical modulation of 5-FU, because little or no advantage is gained by bolus administration (40); in one, the aim is to quickly reach a stable, high concentration by rapid infusion over minutes to hours, whereas in the second, the aim is to extend the duration of exposure by prolonged infusion over hours to weeks. The present study showed that the maximum plasma 5-FU concentration during PMC reached 402 ng/ml, sustaining a concentration of more than 200 ng/ml for 20 h. The efficacy of our regimen convinced us that combination of exposure to continuous low and stable plasma 5-FU concentrations up to the highest possible concentr-
It has been shown that more than 80% of advanced colorectal carcinomas harbor mutations in the p53 tumor suppressor gene (41, 42). In the G1 phase, p53 induces apoptosis or suppresses cell cycle progression through induction of p21WAF1/CIP1, which binds to and inhibits cdk2 and cdk4 (43, 44). However, p21WAF1/CIP1 can also be induced via a p53-independent pathway in several cancer cell lines subjected to genotoxic stress (45, 46). In our study, p21WAF1/CIP1 was induced both in SW480 and HCT116 cells in a time- and dose-dependent manner, regardless of p53 status. To achieve a successful cancer therapy, interactions between these various pathways seem critical for cytotoxicity through apoptosis and cytostasis through G1 arrest.

Recently, Chan et al. (39) demonstrated that 14-3-3σ, a gene originally discovered in differentiating epithelial cells and a member of the 14-3-3 protein family (47–50), is markedly induced by DNA damage in the presence of wild-type p53. Introduction of exogenous 14-3-3σ into cycling cells normally sequesters cyclin B1 and Cdc2 in the cytoplasm, thus preventing the Cdc2–cyclin B complex from entering the nucleus and initiating the transition from G2 to M phase (51). Cytoplasmic localization of cyclin B1 in interphase is determined by an amino acid sequence, referred to as the “cytoplasmic retention signal” (52). Nuclear translocation of cyclin B1 is induced by disruption of the nuclear export signal (NES), a recently identified short leucine-rich sequence (53–56) in the cytoplasmic retention signal region (57). Intriguingly, in mitotic catastrophe seen after treatment with leptomycin B, a specific inhibitor of NES-dependent intracellular transport, cyclin B1 and Cdc2 are not sequestered in the cytoplasm (39, 51, 58). In our study, marked nuclear cyclin B1 accumulation was observed with exposure to 100 ng/ml of 5-FU in SW480, regardless of the status of p53 gene, possibly reflecting mitotic catastrophe. There were significantly fewer apoptotic cells with exposure to 100 ng/ml of 5-FU than 1000 ng/ml in SW480, whereas the growth inhibition was almost the same with both concentrations. Consequently, the majority of the cell death on exposure to 100 ng/ml may be attributed to mitotic catastrophe. These data indicate that colorectal carcinoma cells exposed to 5-FU are targeted to two different pathways, depending on the integrity of their schedule-oriented cell cycle checkpoints; G1-M arrest and mitotic catastrophe at a lower dose (100 ng/ml in SW480 and COLO320DM, and 10 ng/ml in HCT116), and G1–S phase arrest and apoptosis at a higher dose (1000 ng/ml in SW480 and COLO320DM, and 100 ng/ml in HCT116). Actually, apoptotic cells after PMC showed no expression of cyclin B1, which suggests the coexistence of mitotic catastrophe and apoptosis in the surgical specimens (Fig. 5C). This result was consistent with the in vitro data. The point is that the replication-blocked cells do not suffer lethal damage and begin to grow and proliferate again when DNA synthesis is allowed to resume. Enhancement of the 5-FU concentration once a week in combination with a lower, sustained level of 5-FU, thus, seems to contribute to the drastic cytotoxic effect, not only the cytostatic effect, independent of mutations of the p53 gene.

Our results have potentially important clinical implications. The in vitro study showed antitumor effects of 5-FU even in SW480 and COLO320DM cells with mutant p53, although an approximately 10-fold higher concentration of 5-FU was needed to suppress cell growth of these carcinoma cells, compared with HCT116 cells. The antitumor effect of 5-FU via apoptosis has been reported to be markedly reduced if the p53 gene is mutated (59). Moreover, it has been shown that postoperative oral administration of tegafur was effective for the lung cancer patients without p53 mutations but not for those with them (60). Interestingly, it has been reported that the COLO320DM cell line shows many malignant characteristics, including Ras mutations, c-myc amplification, and p53 mutations (61). However, 5-FU was effective even against COLO320DM in this study. Beyond our expectations, the current PMC regimen seems to be efficacious against advanced colorectal carcinoma showing various malignant characteristics. Going forward, it will be possible to modify the regimen in accordance with the malignant potential of each cancer case. Furthermore, a study that showed that the antioxidants pyrroloidinedithiocarbamate and vitamin E can enhance the cytotoxicity of 5-FU and doxorubicin for colorectal carcinoma in a process mediated by induction of p21WAF1/CIP1, but independent of p53 (62), raises another possibility. Development of a new regimen in combination...
with chemotherapeutic agents that target different checkpoints may heighten the efficacy and broaden the selectivity of 5-FU.

The validity of PMC therapy had been proven clearly by two aspects: (a) in vivo evidence of rat models (15); and (b) our clinical data (13, 14), and additionally this study supplied a new aspect. In fact, cell kinetic features in colorectal carcinoma specimens from patients under PMC were consistent with our in vitro study (Fig. 5). However, heterogeneity of the sensitivity to the chemotherapeutic agent is well known to vary in each cell (22). Such heterogeneity, attributable partly to genetic polymorphism or variations, is now
coming into focus in determining appropriate dosage schedules for chemotherapy to obtain maximum effect and minimum toxicity. Clearly, to exploit a more effective regimen, we need to know still more about manipulating checkpoint pathways, making use of DPD or folate-based thymidylate synthase inhibition, and how the pathways are changed in each cancer under PMC. In this regard, the application of immunostaining for cyclin B1, Cdc2, 14-3-3-σ, or p21WAF1/CIP1 in specimens from the patients may be an easy and fairly quick way to evaluate chemosensitivity. Our regimen still leaves room for improvement. We believe that future clinical trials should be directed in the direction of PMC targeting different checkpoints.

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