

Possibility of paclitaxel as an alternative radiosensitizer to 5-fluorouracil for colon cancer

JUNICHIRO HIRO, YASUHIRO INOUE, YUJI TOIYAMA, SHIGEYUKI YOSHIYAMA,
KOJI TANAKA, YASUHIKO MOHRI, CHIKAO MIKI and MASATO KUSUNOKI

Division of Reporative Medicine, Department of Gastrointestinal and Pediatric Surgery, Institute of Life Sciences,
Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan

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Abstract. To evaluate the feasibility of paclitaxel (PTX) radiosensitization for colon cancer, we investigated the cytotoxic and G2/M checkpoint protein (Chk1, Wee1, Bub1, MAD2) effects of 5-fluorouracil (5-FU) or PTX combined with radiation in the human colon cancer cell line LoVo. Cytotoxicity and radiocytotoxicity were evaluated for each drug by the WST-8 colorimetric assay. The IC₂₀ for each drug was determined as a cytotoxic concentration from a survival curve. LoVo cells were exposed to the IC₂₀ of each drug for 24 h and then irradiated. Expressions of the G2/M checkpoint proteins were confirmed by Western blot analysis. Cytotoxicity was induced by 5-FU or PTX alone in a time- and dose-dependent manner. The IC₂₀ of PTX caused higher radiosensitivity than the IC₂₀ of 5-FU ($P < 0.05$). Western blot analysis revealed different expression patterns of the G2/M checkpoint proteins between 5-FU and PTX pre-treatments. 5-FU combined with radiation tended to decrease the expressions of all G2/M checkpoint proteins in a time-dependent manner. PTX combined with radiation maintained high expressions of Chk1 and MAD2 proteins for 24 h post-radiation and, in particular, MAD2 protein was strongly induced by PTX with high-dose radiation. PTX showed higher radio-sensitization than 5-FU for the colon cancer cell line LoVo and may be an alternative radiosensitizer to 5-FU in the clinical setting.

Introduction

Chemoradiotherapy is now widely used as the definitive and adjuvant therapy for the majority of cancer patients. Randomized trials have shown that such combined treatments

improve survival compared with radiation alone in locally advanced cancers of the head and neck, lung, esophagus, stomach and rectum (1-5). Despite these resounding clinical successes, the mechanisms by which conventional chemotherapeutic agents produce radiosensitization remain largely unknown. Recently, the interest in preoperative chemoradiotherapy for resectable rectal cancer has increased, because it has the advantage of enhancing local-regional control by eliminating microscopic residual disease around the primary tumor and in the draining lymphatics, with a possible positive impact on overall survival (5).

5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents for colorectal cancer and has been extensively used with radiation. There are a number of mechanisms by which 5-FU could increase radiosensitivity at the cellular level. 5-FU-induced radiosensitivity is thought to be responsible for the killing of S phase cells, which are relatively radioresistant (6). However, this effect cannot account for all of the increase in radiosensitivity produced by the drug. Several studies have suggested that 5-FU should be given continuously during a course of fractionated radiation if radiosensitization is to be achieved (7-9).

On the other hand, paclitaxel (PTX) is known to be one of the most active cancer chemotherapeutic agents. It is effective against a variety of human tumors, including ovarian, breast, head and neck, and non-small cell lung cancers (10-13). Unlike 5-FU, PTX interferes with mitotic spindle function by enhancing the rate and yield of microtubule assembly and preventing microtubule depolymerization, resulting in G2/M arrest (14). Since the G2/M phase is the most radiosensitive phase of the cell cycle, G2/M arrest induced by PTX provides a biological rationale for testing PTX/radiation combination treatments *in vitro* (15). Preliminary *in vitro* studies have suggested that PTX is a useful radiosensitizing agent in a variety of tumor cell lines (16,17). Interestingly, PTX is even considered to be a radiosensitizer at low doses. However, in light of the controversial experimental data, the existence and extent of its effect at low doses is the subject of intense debate (18,19). Although, clinical trials have unfortunately demonstrated that PTX as a single agent does not show activity in colorectal cancer (20), we were interested in the function of PTX as a cell cycle regulator and hypothesized that it may be an alternative radiosensitizer to 5-FU for colorectal cancer, especially in cases where the aim of the concurrent chemotherapy is enhancement of local control rather than systemic

Correspondence to: Dr Masato Kusunoki, Division of Reporative Medicine, Department of Gastrointestinal and Pediatric Surgery, Institute of Life Sciences, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan
E-mail: kusunoki@clin.medic.mie-u.ac.jp

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control. To confirm this hypothesis, we simultaneously evaluated the radiosensitizing mechanisms of 5-FU and PTX for colon cancer, from the viewpoint of cell cycle.

The present study aimed to investigate the cytotoxic and G2/M checkpoint protein effects of 5-FU or PTX combined with radiation in a human colon cancer cell line and to evaluate the feasibility of PTX radiosensitization for colon cancer.

Materials and methods

Cell culture. A wild-type p53-expressing human colon adenocarcinoma cell line, LoVo, was obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Tokyo, Japan). LoVo cells were grown in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with fetal bovine serum [10% (v/v); Gibco BRL, Tokyo, Japan], glutamine (2 mM), penicillin (100000 U/l) and streptomycin (100 mg/l) at 37°C in a 5% CO₂ incubator.

Anticancer agents. 5-FU and PTX were obtained from Sigma-Aldrich, reconstituted in distilled water at appropriate concentrations and stored at -20°C until use.

Experimental protocol. Although the IC₂₀ (drug concentration responsible for 20% cell growth inhibition) of each drug was used for this study, we used clinical concentrations as much as possible. The 5-FU concentrations were chosen based on both our previous reports (21-23) and drug information obtained from Kyowa Hakko Kogyo (Tokyo, Japan). The drug information for 5-FU indicated that its plasma concentrations reach 15.3 µg/ml (100 µM) after a bolus injection of 5-FU (500 mg/m²) and 0.6 µg/ml (5 µM) during continuous infusion of 5-FU (60 mg/kg/48 h). The PTX concentrations were chosen based on the plasma concentrations obtained from clinical use cited in the drug information for PTX (Taxol; Bristol Myers Squibb, Tokyo, Japan). This information indicated that the plasma concentrations of PTX reach 1-10 µg/ml (1-10 µM) after an injection and 0.05-0.1 µg/ml (50-100 nM) at 24 h after drip infusion of PTX (105-270 mg/m²). To elucidate the irradiation effects in detail, irradiation was carried out at different doses (2.5 and 5 Gy). All irradiation treatments were performed with a CLINAC 2100C X-ray system (Varian Oncology Services, USA) at 4 MV, using a 40-mm solid water phantom and a dose rate of 217 cGy/min.

Drug concentrations, irradiation and administration schedules. As mentioned above, we adopted clinically relevant concentrations of 5-FU and PTX in this study. Although we should ideally have considered the doubling time of LoVo cells before deciding the exposure time, we chose to use an exposure of 24 h for each drug for experimental simplicity. The final concentrations ranged from 0.1-1000 µM for 5-FU and 0.001-10 µM for PTX. The drug exposure and irradiation schedules are summarized in Fig. 1. To test the cytotoxicity of each drug, LoVo cells in the exponential growth phase were treated with various concentrations of 5-FU or PTX for 24 h. After discarding the medium containing each drug and replacing it with fresh medium, the cytotoxicity was evaluated using a 2-(2-methoxy-4-nitrophenyl)-3-(4-

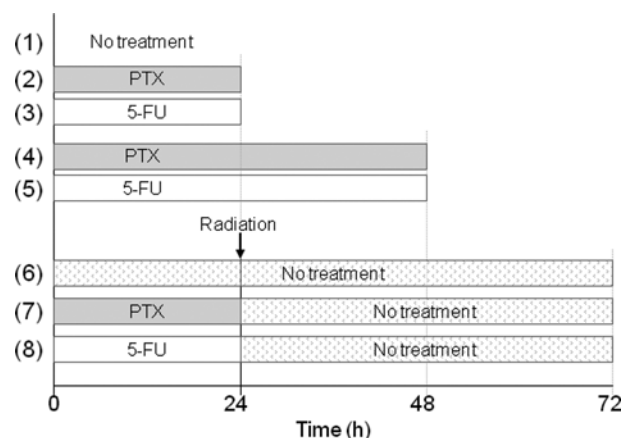


Figure 1. Drug administration and irradiation schedules used in this study. (1) No treatment, comprising control cells. (2) and (3) drug exposure for 24 h. (4) and (5) drug exposure for 48 h. (6) No drug treatment or irradiation, comprising radiation control cells. (7) and (8) irradiation after 24 h of drug exposure (medium replaced with fresh medium).

nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) colorimetric assay. For irradiation experiments, LoVo cells were treated with each drug at its IC₂₀ for 24 h. After removing the drugs from the wells and refilling the wells with fresh medium, irradiation was carried out at different doses. The irradiated cells were incubated for 0, 24 or 48 h, and the cytotoxicity was evaluated using the WST-8 colorimetric assay.

Growth inhibition assay. Cytotoxicity was evaluated by the WST-8 colorimetric assay using a Cell Counting kit (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. Cells (5x10³) were seeded into 96-well plates (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) in 100 µl of culture medium for 24 h prior to drug exposure, and then treated with various concentrations of 5-FU or PTX for various durations. Cell viability was determined colorimetrically by the optical density at a wavelength of 450 nm using a microplate reader (SoftMax; Molecular Devices, Sunnyvale, CA, USA). The percent cell survival for each drug concentration was calculated using the following formula: (absorbance of test wells/absorbance of control wells) x 100.

Western blot analysis. At 0, 12, 24, 36 and 48 h after irradiation, cells were harvested and subjected to immunoblotting analysis. The cells were homogenized in lysis buffer (Tris-buffered saline, pH 7.5, containing 2% Triton X-100) for 5 min on ice. The protein concentration was measured by the BCA protein assay (Pierce, Rockford, IL, USA). Lysates containing 20 µg of total protein were mixed with an equal volume of 2X Laemmli loading buffer containing 2-mercaptoethanol and heated at 100°C for 5 min. The samples were electrophoretically separated in 12.5% gradient polyacrylamide gels containing 0.1% SDS at 25 mA for 2 h, followed by semi-dry transfer to Immobilon-PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 12 V for 2 h. The membranes were blocked with 2% skimmed milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween-20. The primary antibodies

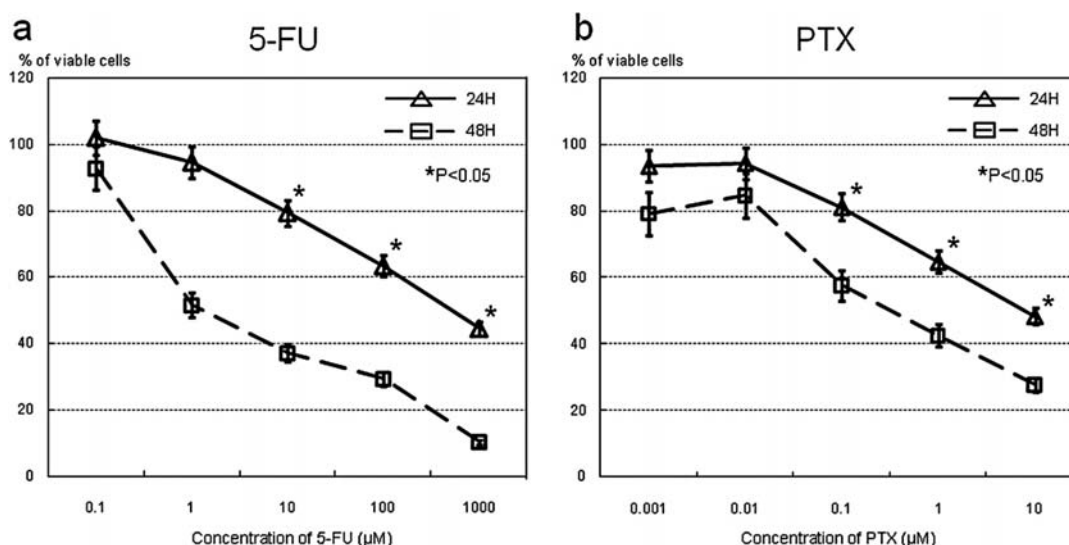


Figure 2. Cytotoxicities of 5-FU and PTX toward LoVo cells. Cells were treated with different concentrations of 5-FU (A) and PTX (B) for 24 (triangles) or 48 h (squares). Cell growth was determined using a WST-8 colorimetric assay. The results are expressed as the percentage of cell growth relative to untreated control cells. The data represent the means \pm SD of three experiments.

used were: mouse monoclonal anti-hsMAD2 (1:1000 dilution; Transduction Laboratories, Lexington, KY, USA); mouse monoclonal anti-Wee1 (1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-Chk1 (1:400 dilution; Santa Cruz Biotechnology); mouse monoclonal anti-Bub1 (1:400 dilution; Chemicon International, Temecula, CA, USA); and mouse monoclonal anti-actin (clone C4; 1:3000 dilution; ICN Biomedicals, Aurora, OH, USA). The secondary antibody used was alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, Madison, WI, USA) diluted 1:1000. Following treatment with an enhanced chemiluminescence detection solution, the membranes were exposed to X-ray film for autoradiographic visualization. The films were scanned and the relative quantities of the protein bands were analyzed by densitometry using CS Analyzer version 2.0 (ATTO Corp., Tokyo, Japan).

Statistical analysis. The results are expressed as means \pm SD. The Mann-Whitney U test was used for comparisons between unpaired groups. Values of $P < 0.05$ were considered statistically significant. Stat View analysis software (version 5; Abacus Concepts, Inc., Berkeley, CA) was used for all analysis.

Results

Cytotoxic effects of 5-FU and PTX. Growth-inhibitory effects were observed for treatment with either 5-FU or PTX alone in a time- and dose-dependent manner (Fig. 2). LoVo cell growth was inhibited at the clinically used concentrations of 5-FU (0.1–10 μ M) and PTX (0.001–10 μ M). The IC₂₀ values for 5-FU and PTX were 10 and 0.1 μ M, respectively.

Radiocytotoxic effects of 5-FU and PTX. To examine whether 5-FU or PTX can target LoVo cells and confer radiosensitivity, cells were treated with the IC₂₀ values of 5-FU (10 μ M) or PTX (0.1 μ M) for 24 h. After removing the drugs, the cells were irradiated at a dose of 2.5 or 5 Gy. Growth inhibition

was measured using the WST-8 colorimetric assay. The results are shown in Fig. 3. In all groups, radiocytotoxic effects were seen in a time- and radiation dose-dependent manner. PTX pretreatment was found to have the highest radiosensitivity enhancement effect for both radiation doses. Fig. 3a shows the radiosensitivity effects of 5-FU and PTX with low-dose radiation (2.5 Gy). PTX pretreatment caused a higher radiosensitivity enhancement effect than 5-FU for 48 h after individual pretreatment ($P < 0.05$). Interestingly, PTX, but not 5-FU, showed a radiosensitivity enhancement effect at 24 h after pretreatment. Fig. 3b shows the radiosensitivity effects of 5-FU and PTX with high-dose radiation (5 Gy). The maximum effect was seen when 0.1 μ M PTX was combined with 5 Gy of irradiation. PTX pretreatment showed a higher radiocytotoxic effect than 5-FU pretreatment and radiation alone at both 24 and 48 h after pretreatment ($P < 0.05$). We subsequently investigated the relationships of these radiosensitizing effects with cell cycle protein expressions.

Western blot analysis. The results of Western blot analysis are shown in Fig. 4. Radiation alone at 2.5 or 5 Gy showed similar expression patterns of G2/M-related proteins (Fig. 4a). The expressions of the Chk1 and Wee1 proteins in the control cells treated with radiation alone increased until 24 h after irradiation and then returned to their basal levels. Bub1 protein expression was decreased in a time-dependent manner by irradiation at 2.5 Gy, but was not affected by irradiation at 5 Gy. MAD2 protein expression gradually increased until 24 h after 2.5-Gy irradiation and 36 h after 5-Gy irradiation.

After PTX or 5-FU pretreatment, we observed wide differences in the expressions of some proteins (Fig. 4b, c). 5-FU pretreatment tended to decrease the expressions of all G2/M checkpoint proteins in a time-dependent manner. In contrast, PTX pretreatment maintained Chk1 and MAD2 protein expressions at high levels for 24 h post-irradiation and, in particular, MAD2 protein expression was strongly induced by 5-Gy radiation when the maximum radiocytotoxic effect

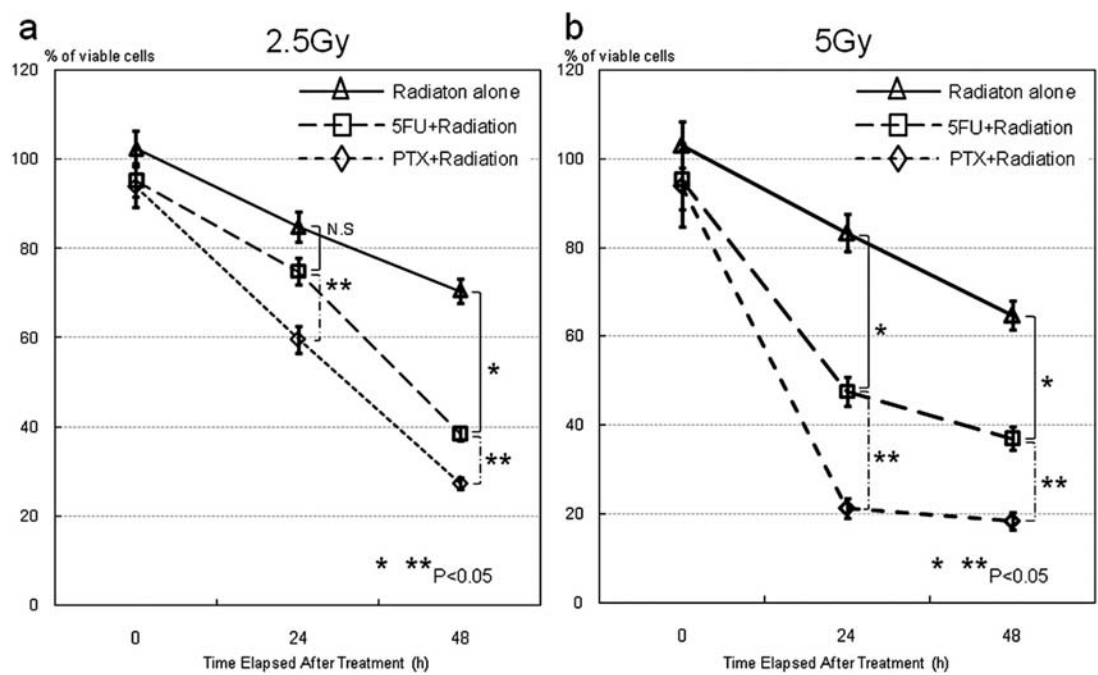


Figure 3. Radiocytotoxic effects of 5-FU and PTX on LoVo cells. (A), (B) LoVo cells were pretreated with 5-FU (squares) or PTX (diamonds) at their IC₂₀ values for 24 h and then irradiated with 2.5 (A) or 5 (B) Gy. Other cells were treated with radiation alone (triangles). Cell growth was determined using a WST-8 colorimetric assay. The results are expressed as the percentage of cell growth relative to untreated control LoVo cells. The data represent the means \pm SD of three experiments.

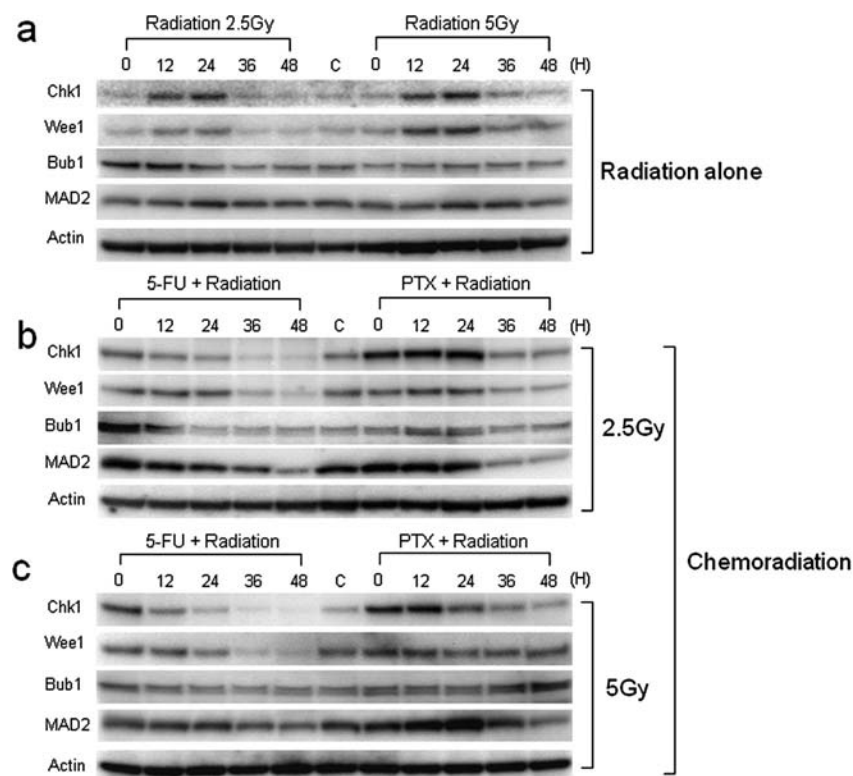


Figure 4. Western blot analyses of Chk1, Wee1, Bub1, MAD2 and actin in LoVo cells. (A) Cells treated with radiation alone (2.5 or 5 Gy). (B) Cells treated with 2.5 Gy of irradiation after pretreatment for 24 h with 5-FU or PTX at their IC₂₀ values. (C) Cells treated with 5 Gy of irradiation after pretreatment for 24 h with 5-FU or PTX at their IC₂₀ values.

was obtained. Furthermore, at 24 h after 2.5-Gy irradiation, which showed radiosensitization enhancement by PTX but not 5-FU, PTX pretreatment clearly induced Chk1 and Bub1 protein expressions, unlike 5-FU.

Discussion

In general, cells are most sensitive to irradiation during mitosis in the G₂ phase, less sensitive in the G₁ phase and least sensi-

tive during the latter part of the S phase (24). Since various types of chemotherapeutic agents are able to arrest cells at specific cell-cycle checkpoints, previous studies have explored the use of different chemotherapeutic agents to synchronize and arrest cells in the radiosensitive phases of the cell cycle (25,26). 5-FU is an analog of uracil, which is converted intracellularly into metabolites that inhibit the enzyme thymidylate synthase, thereby preventing DNA, RNA and protein synthesis (6,7). 5-FU has been the most commonly used chemotherapeutic agent in the clinical treatment of colorectal cancer. Radiosensitization by 5-FU is thought to increase radiosensitivity at the cellular level by killing cells in the S phase, which are relatively radioresistant (6). However, 5-FU-induced radiosensitization cannot be completely explained by redistribution of the cells into a radiosensitive phase of the cell cycle. The mechanism of 5-FU-induced radiosensitization remains largely unknown at the cellular level. Meanwhile, PTX is characterized by specific inhibition of microtubule depolymerization, thereby causing G2/M phase accumulation and mitotic arrest of tumor cells (27,28). *In vitro*, PTX has the exceptional property of causing cancer cell death independently of wild-type p53. PTX-mediated blockade at the G2/M phase can activate cell-cycle control pathways to induce apoptosis independently of p53 (29,30). PTX also enhances the cytotoxic effects of ionizing radiation on pancreatic, breast, ovarian, and head and neck cancer cell lines, possibly by inducing arrest at the G2 and mitotic phases of the cell cycle, which are the most radiosensitive (16,17,31,32). However, the Eastern Cooperative Oncology Group trial demonstrated that PTX as a single agent does not have activity against colon or rectum adenocarcinoma (20). Therefore, in colorectal cancer treatment, PTX is rarely used and has not been sufficiently evaluated.

In the present study, we investigated the biological effects of optimal 5-FU or PTX treatment and irradiation on the human LoVo colon cancer cell line. The drug concentrations used were calculated according to those used in clinical situations. To simulate a clinical setting, we used the IC20 of each drug as the minimum cytotoxic concentration and examined their effects in combination with 2.5 and 5 Gy of irradiation. In addition, we investigated the expression patterns of major G2/M-related proteins (Chk1, Wee1, Bub1 and MAD2). Chk1 is thought to play a central role in G2 phase arrest (33,34). Entry into mitosis requires activation of Cdc2 following removal of inhibitory phosphates by Cdc25. Cdc2 kinase activity is regulated in an opposing manner by the kinase Wee-1 (35,36). MAD2 and Bub1 play central roles in the mitotic checkpoint and induce G2/M phase arrest. Bub1 is involved in recruiting other checkpoint proteins to unattached kinetochores, which subsequently activate MAD2, leading to suppression of anaphase-promoting complex formation and halting entry into anaphase (29,30).

Our present results revealed that PTX pretreatment had a significantly higher radiosensitizing effect than 5-FU at both the doses of radiation examined. Previous studies involving flow cytometric analyses of LoVo cells demonstrated that 24 h of exposure to 10 μ M 5-FU increases the number of S-phase cells among the treated cells compared with control cells, while 24 h of exposure to 0.1 μ M PTX induces accumulation of tumor cells in the G2/M phase (18,37). These findings suggest that high radiosensitivity may be derived from

radiation exposure under the presence of G2/M phase-accumulated cells induced by PTX. The present study also revealed that the expression patterns of G2/M-related proteins following treatment with PTX and radiation differed significantly from those of 5-FU and radiation, and that these effects were especially dependent on the radiation dose. The combination of PTX and low-dose radiation may result in a large and prolonged blockade of the G2/M cell cycle phase and the cancer cells may become arrested at the G2/M checkpoint to repair radiation-induced DNA damage. Furthermore, MAD2 protein expression was strongly induced by 5 Gy of irradiation when the maximum radiocytotoxic effect was obtained. This observation suggests that the combination of PTX and high-dose radiation may induce mitotic catastrophe, since several studies have demonstrated the occurrence of PTX-induced mitotic cell cycle arrest or MAD2 protein expression (29,30,38,39).

In conclusion, low-dose PTX showed higher radiosensitization than 5-FU for LoVo colon cancer cell line cells. PTX may have potential as an alternative radiosensitizing agent to 5-FU for colon cancer, although prospective clinical trials are needed to determine the actual benefits.

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