prevention and treatment of chemotherapy-induced emesis has enabled many patients to achieve good control of this side-effect[3].

Granisetron is a potent and selective antagonist at the 5-HT_3 receptor[10]. Clinical trials have shown that it controls nausea and vomiting in patients undergoing moderately or severely emetogenic chemotherapy[9,11], while granisetron given with a corticosteroid enhances antiemetic efficacy further[9]. However, concomitant administration of supportive care agents with antineoplastic drugs increases the risk of drug–drug interactions via the cytochrome P450 (CYP) system[12]. Furthermore, the majority (60%) of cancer patients are aged 65 years and over[13] and approximately 80% of them have comorbid conditions for which they are taking prescription medications[14,15]. A recent study in patients with advanced cancer indicated that the potential for drug–drug interactions increases with age, number of concomitant medications and length of hospital stay[16]. Although all the patients in the study were suffering from advanced cancer, and may have been experiencing specific problems related to the stage of their disease, the study indicates that there is considerable potential for drug–drug interactions in elderly patients with cancer. This may compromise treatment efficacy or increase drug-related toxicity.

Doxorubicin is metabolized to doxorubicinol by cytoplasmic aldo-keto reductase, and both doxorubicin and doxorubicinol are further metabolized by CYP enzymes[17]. The major metabolic pathway of etoposide is 3′-demethylation by CYP3A4[18], while oxidation of the piperidinylpiperidine side chain of irinotecan by CYP3A4 produces the relatively inactive oxidative metabolites 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin[19]. The metabolism of granisetron is also mediated via the CYP3A subfamily to produce the major metabolite 7-hydroxygranisetron and also a smaller amount of 9′-desmethylgranisetron[20]. The role of the CYP3A isoenzymes in the metabolism of granisetron, therefore, raises the potential for drug–drug interactions between granisetron and any other agent metabolized through the same enzyme pathway.

Granisetron does not appear to inhibit the activities of CYP1A2, CYP2A6, CYP2B6, CYP2C9/8, CYP2C19, CYP2D6, CYP2E1 or CYP3A at concentrations up to 250 μM[20]. Furthermore, experience from our laboratory suggests that granisetron has no effect on the *in vitro* metabolism in human liver microsomes of either paclitaxel or docetaxel, both of which are also metabolized by CYP3A4[21]. To further evaluate the potential for granisetron interactions with chemotherapeutic agents, the present study investigated the effects of granisetron on the *in vitro* metabolism of doxorubicin, irinotecan and etoposide in human liver microsomes.

Methods

Chemicals

Granisetron was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Irinotecan and SN-38, a potent inhibitor of topoisomerase I, were obtained from Yakult Honsha Co., Ltd. (Tokyo, Japan). Doxorubicin, etoposide, (S)-(+)-camptothecin, ethyletoposide, daunorubicin and quercitrin were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and ketoconazole was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). All other reagents were purchased from commercial sources and were of the highest grade.

Human liver microsomes

Four batches of pooled human liver microsomes (International Institute for the Advancement of Medicine: IIAM, Scranton, PA, USA), collected from 15 donors, were used. They were preserved at –80°C until the time of use.

Analytical procedures

Human liver microsomes, 250 μg, were incubated at 37°C in the presence of 0.1 M potassium phosphate buffer (pH 7.4) and 0.1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a final volume of 0.5 mL. Preliminary experiments determined the incubation time and the concentration of doxorubicin, irinotecan and etoposide. Based on the findings of these studies, granisetron (final concentration 20 nM, 200 nM and 2000 nM) was incubated with the human liver microsome preparation in combination with either doxorubicin, 20 μM, for 4 h, irinotecan, 10 μM, for 60 min or etoposide, 50 μM, for 2 h. The levels of unchanged doxorubicin, irinotecan and etoposide in the incubation mixtures were determined by high-performance liquid chromatography (HPLC). Ketoconazole, 20 μM, a potent inhibitor of CYP3A4, served as a positive control for irinotecan and etoposide, while quercitrin, 2 mM, a potent inhibitor of aldo-keto reductase, served as a positive control for doxorubicin.

HPLC conditions

The LC-10A System (Shimadzu Co., Tokyo, Japan) was used as the determination device with an Inertsil separation column (150 × 4.6 mm internal diameter, 5 μm, GL Sciences Inc., Tokyo, Japan) with a guard column (Guard Pal Inserts Nova Pak C18, Waters Co., Milford, CT, USA).

HPLC for determination of doxorubicin was performed according to the method of de Bruijn *et al.*[22]. The flow

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rate of the mobile phase was set at 1.25 mL/min, column temperature was 50°C and the wavelength for UV detection was 480 nm. Retention times for unchanged doxorubicin and daunorubicin in the human liver microsome preparations were 8.5 min and 22 min, respectively, and the concentrations of doxorubicin and daunorubicin were calculated using the area under the peak. The limit of detection for doxorubicin was 0.3 µM.

HPLC for determination of irinotecan was performed according to the method of Haaz *et al.*[23]. The flow rate of the mobile phase was set at 1.0 mL/min, column temperature was room temperature and the wavelength for UV detection was 355 nm. Retention times for unchanged irinotecan, its active metabolite SN-38 and camptothecin in the human liver microsome preparations were 17 min, 29 min and 28 min, respectively, and the concentrations of irinotecan, SN-38 and camptothecin were calculated using the area under the peak. The limit of detection for irinotecan was 0.3 µM.

HPLC for determination of etoposide was performed according to the method of Kawashiro *et al.*[18]. The flow rate of the mobile phase was set at 1.0 mL/min, column temperature was room temperature and the wavelength for UV detection was 288 nm. Retention times for unchanged etoposide and ethyletoposide in the human liver microsome preparations were 8.5 min and 16 min, respectively, and the concentrations of etoposide and ethyletoposide were calculated using the area under the peak. The limit of detection for etoposide was 1.5 µM.

Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple t-test. A value of $p < 0.05$ was classed as significant.

Results

All data are the mean ± SE of four determinations.

Doxorubicin

Preliminary experiments established optimum conditions of 4-h incubation time with doxorubicin concentrations of 10 µM (Figure 1A). In the presence of NADPH, quercitrin, 2 mM, inhibited aldo-keto reductase metabolism and prevented the breakdown of doxorubicin in the human microsomal preparations (Figure 2A). In the absence of granisetron, the amount of unchanged doxorubicin (10 µM) in the incubation mixture decreased by 34.2 ± 5.5% (Figure 2A). Granisetron, 20 nM, 200 nM and 2000 nM, had no effect on the rate of doxorubicin reduction ($p > 0.05$; Figure 2A).

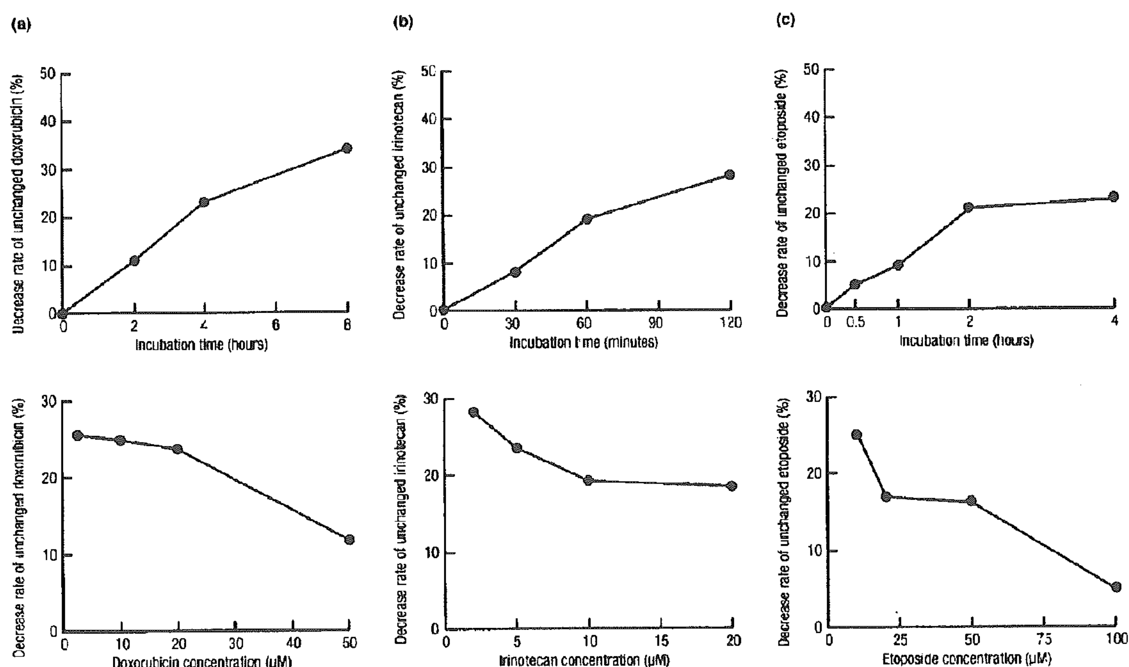

Figure 1. Effect of incubation time on metabolism (upper panel) and concentration in human liver microsomes (lower panel) of (a) doxorubicin, (b) irinotecan and (c) etoposide

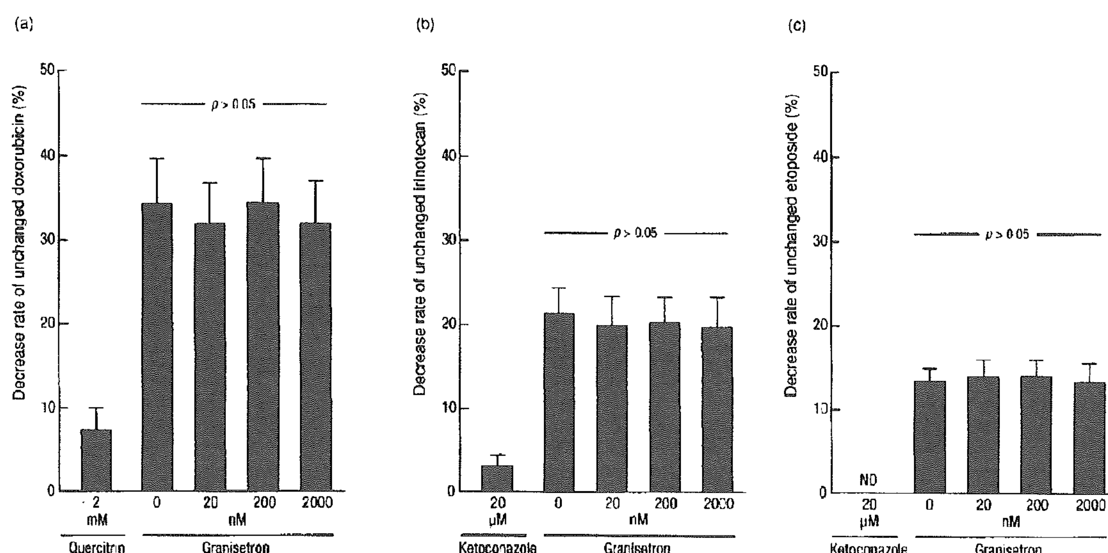

Figure 2. Effect of granisetron on the metabolism of (a) doxorubicin, (b) irinotecan and (c) etoposide in human liver microsomes. Data are the mean ± SE of 4 determinations (ND, not detected; $p > 0.05$ *vs no granisetron)*

Irinotecan and etoposide

Optimum conditions established by preliminary experiments were 60-min incubation time with concentrations of irinotecan, 10μM (Figure 1B), and 2-h incubation time with concentrations of etoposide, 50μM (Figure 1C). In the presence of NADPH, ketoconazole, 20μM, inhibited CYP3A4 metabolism and prevented the breakdown of irinotecan (Figure 2B) and etoposide (Figure 2C) in the human microsomal preparations. In the absence of granisetron, the amount of unchanged irinotecan (10μM) in the incubation mixture decreased by 21.3 ± 2.9% (Figure 2B), while that of unchanged etoposide (50μM) decreased by 13.4 ± 1.6% (Figure 2C). Granisetron, 20nM, 200nM and 2000nM, had no effect on the rates of reduction of irinotecan ($p > 0.05$; Figure 2B) or etoposide ($p > 0.05$; Figure 2C).

Discussion

The 5-HT_3-receptor antagonist, granisetron, is metabolized primarily via the CYP3A isoenzymes. Bloomer *et al.* have previously demonstrated that granisetron at concentrations between 0.5μM and 250μM did not inhibit the activity of a number of CYP isoenzymes[20], and the present study has confirmed that it neither inhibits nor induces the enzymes involved in the metabolism of doxorubicin, irinotecan and etoposide in an *in vitro* human liver microsomal preparation. These results further extend the knowledge from a previous study, which found that granisetron did not alter the *in vitro* metabolism of docetaxel or paclitaxel in human liver microsomes[21]. In the present study, granisetron did not interact with the breakdown of doxorubicin, irinotecan or etoposide, even when present at concentrations up to 60-fold higher than the maximum plasma concentrations that have been reported *in vivo*[24].

Ketoconazole is known to be a potent, selective inhibitor of the CYP3A isoenzymes, with a K_i of less than 1μM[25]. Consistent with this, the present study found that ketoconazole, 20μM, was a potent inhibitor of the metabolism of irinotecan and etoposide, both of which are metabolized by CYP3A4. Additionally, quercitrin, which is an inhibitor of aldo-keto reductase, completely inhibited the reduction of doxorubicin. In contrast, granisetron had no effect on the metabolism of the three study agents.

Sixty per cent of cancer patients are classed as elderly (over 65 years of age) and, as such, have an increased risk of suffering from declining organ function and a number of comorbid conditions[13,14]. As a result, they will often consume multiple medications[6]: indeed, 78% of patients aged over 65 years are estimated to be taking prescription medications, while 39% consume five or more drugs[15]. The risk of drug–drug interactions has been shown to increase with age and concomitant medications[16], and is estimated to be over 50% when a patient is receiving five medications, rising to a 100% probability with seven or more medications[26]. It is, therefore, essential that the potential for drug–drug interactions is minimized through identification of offending agents or the use of agents with no known drug–drug interactions.

Current antiemetic guidelines recommend the prophylactic administration of a 5-HT_3-receptor antagonist

in combination with dexamethasone and aprepitant for highly emetogenic chemotherapy, and a 5-HT_3-receptor antagonist in combination with dexamethasone for moderately emetogenic chemotherapy[27]. Among the currently available 5-HT_3 antagonists, granisetron is metabolized exclusively by the CYP3A isoenzymes. In contrast, ondansetron is metabolized by CYP2D6, CYP1A2 and to a small extent CYP1A1, in addition to the CYP3A isoenzyme[12]. Consequently, there is a potential for multiple drug–drug interactions between ondansetron and other agents that are also metabolized by any of these isoenzymes, and there have been a number of documented drug–drug interactions with this agent[28–31]. Furthermore, dolasetron and tropisetron are also both metabolized in part by members of the CYP3A group, in addition to CYP2D6. This is of particular concern given that CYP2D6 is subject to genetic polymorphism[32,33].

As doxorubicin, irinotecan and etoposide are all associated with the debilitating side-effects of nausea and vomiting, an antiemetic agent is indicated for patients undergoing treatment with any of these drugs. However, consideration must be given to the choice of antiemetic agent and its potential for drug–drug interactions. The present study suggests that granisetron can be used to treat nausea and vomiting induced by antineoplastic regimens that include doxorubicin, irinotecan or etoposide, with minimal risk of drug–drug interactions.

Conclusions

This study indicates that the 5-HT_3-receptor antagonist granisetron neither inhibits nor induces enzymes involved in the metabolism of doxorubicin, irinotecan and etoposide. The use of granisetron for the treatment of nausea and vomiting should, therefore, limit the potential for drug–drug interactions.

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Evaluation of weekly low-dose paclitaxel and carboplatin treatment for patients with platinum-sensitive relapsed ovarian cancer

Yoh Watanabe*, Hidekatsu Nakai, Haruhiko Ueda, Hiroshi Hoshiai

Department of Obstetrics and Gynecology, Kinki University School of Medicine, Osaka, Japan

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Abstract

Objective. Although paclitaxel and carboplatin therapy (TC) is an established effective standard regimen for patients with ovarian cancer, both treatment delay for hematologic toxicity and discontinuation of treatment due to neurotoxicity have occasionally been reported. To achieve therapeutic density, we evaluated the usefulness of weekly low-dose TC therapy (WTC) in patients with platinum-sensitive (median PFI was 11.4 ± 2.7 months) recurrent epithelial ovarian cancer.

Methods. A total of 25 patients were treated with paclitaxel at 60 mg/m^2 and carboplatin at AUC = 2 using 3 weekly courses with a 1-week break schedule. Eighteen patients had assessable tumors for response, and the other seven patients were evaluated by CA-125-based response. All of the patients were assessable for toxicity.

Results. The overall response rate (OR) based on WHO criteria was 84.2% (95% CI; 0.65–0.98), including nine complete responses (CR); OR based on CA-125 was 85.7% (95% CI; 0.42–0.99), including 3 CR. The total response rate was 88.0% (95% C.I.; 0.68–0.97). The median progression-free survival of the patients was 13.5 months during the mean follow-up period of 21.9 ± 9.2 months. No patients had grade 1 or higher thrombocytopenia, and although 44% of the patients developed neurotoxicity, all cases remained grade 1. Treatment delay of over 7 days due to toxicity was observed in only two patients (16.0%) and in six cycles (1.3%) in a total of 451 cycles.

Conclusion. WTC combination, as used in this study, produced a high response rate with acceptable toxicity, and the optimal combination in a weekly regimen remains to be determined.
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Keywords: Weekly administration; Paclitaxel; CBDCA; Recurrent ovarian cancer

Introduction

Recently, the ICON 4 trial [1] demonstrated that paclitaxel/platinum therapy was significantly superior in achieving progression-free survival (PFS) and overall survival compared to those achieved by conventional nonpaclitaxel/platinum chemotherapy in patients with platinum-sensitive recurrence. Therefore, paclitaxel and carboplatin (CBDCA) therapy (TC) is being developed as an effective regimen not only for patients with primary epithelial ovarian cancer but also for platinum-sensitive relapsed epithelial ovarian cancer. However, with treatment by TC therapy, thrombocytopenia, alopecia, and neurotoxicity are frequently experienced as uncontrolled adverse effects. In our department, several patients rejected continuation of TC therapy due to neurotoxicity, and several patients rejected even starting TC therapy due to their anxiety about neurotoxicity. These trends indicate that thrombocytopenia, alopecia, and neurotoxicity, especially sensory neurotoxicity, should be resolved while maintaining therapeutic effects, dose intensity, and providing an incentive for treatment and maintaining the patient's quality of life. Recently, several studies reported the efficacy of weekly 1-h paclitaxel infusion therapy for advanced or recurrent cancer.

* Corresponding author. Department of Obstetrics and Gynecology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osakasayama, Osaka 589-8511, Japan. Fax: +81 72368 3745.
E-mail address: watanabe@med.kindai.ac.jp (Y. Watanabe).

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Weekly administration regimens afford theoretical advantages based on cell cytokinetic effects of drug-resistant clones by frequent exposure of cancer cells to anti-neoplatic agents [2], while the weekly schedule was also expected to offer less toxicity than the standard schedule. Seidman et al. [3] treated 30 patients with metastatic breast cancer with weekly paclitaxel infusion at an initial dose of 100 mg/m^2 until disease progression, and reported that the OR was 53%, the complete response rate was 10%, 13.3% of grade 3/4 neutropenia, and 9.5% grade 3 neuropathy. They concluded that weekly paclitaxel infusion therapy will be an attractive treatment alternative for patients with metastatic breast cancer due to its high therapeutic index, manageable toxicity, and convenient administration schedule. As a second-line chemotherapy for relapsed ovarian cancer, Abu-Rustam et al. [4] studied the effects of weekly paclitaxel (60–100 mg/m^2) as salvage therapy for relapsed ovarian cancer, and reported an OR of 28.9%. Moreover, Markman et al. [5] evaluated a phase II study of weekly single-agent at 80 mg/m^2 of paclitaxel, and reported that the OR was 25%. Recently, Havrilesky et al. [6] performed a phase II study of WTC (T: 80 mg/m^2, C: AUC = 2) in recurrent ovarian and peritoneal cancers, and reported an 82.8% OR and a 13.7-month median interval until progression in platinum-sensitive patients and an 11.5-month overall median interval until progression in platinum-resistant patients. Moreover, Wu et al. [7] also reported the results of WTC (T: 60 mg/m^2, C: AUC = 2) for Chinese patients with advanced ovarian cancer in comparison with those on the monthly regimen. They reported that WTC achieved 71.4% OR, and although there were no significant differences in nonhematological toxicities between WTC and the monthly regimen, delayed treatment, unanticipated hospitalization, and G-CSF support were much less frequent with WTC. Weekly fractionated platinum for recurrent ovarian cancer has also been studied by several groups [8–10]. Colombo et al. [9] and Cocconi et al. [10] conducted randomized trial, compared weekly cisplatin to every-3-week dosing, and reported the potential role for weekly platinum therapy for recurrent ovarian cancer. Recently, Belani et al. [11] reported that weekly paclitaxel with nonfractionated CBDCA administration achieved the superior effects than weekly paclitaxel with fractionated CBDCA in patients with stage IIIB or IV non-small-cell lung cancer. According to those reports, although the addition of CBDCA will be needed to achieve a better response rate, in the chemotherapy for advanced non-small-cell lung cancer, it has been thought that nonfractionated CBDCA administration was more favorable in weekly TC regimen. However, in recurrent epithelial ovarian cancer, phase II study of WTC with nonfractionated CBDCA was just now underway, and only overall response was reported [Rose et al., Proc Am Soc Clin Oncol 2003, Abstr. 1932]. Moreover, prior studies of WTC with fractionated CBDCA [6,7] [Dunton CJ et al., Proc Am Soc Clin Oncol 2003, Abstr. 1876] have achieved satisfactory effects both in the OR and the PFS. According to the results of those trials, the optimal dose and schedule for WTC therapy for recurrent ovarian cancer remain unknown. Furthermore, there is some possibility that the clinical role of fractionated CBDCA administration would be different in chemosensitive tumor, such as epithelial ovarian cancer. Thus, we have conducted phase II study and pharmacokinetic study of WTC using fractionated CBDCA subjected the same as in the recent reports [6,7] from 2000 in Japanese patients with platinum-sensitive recurrent ovarian cancer for further comparative study to evaluate the clinical role of WTC.

Patients and methods

Treatment regimen

From October 2000 to August 2003, 25 patients with relapsed epithelial ovarian cancer who had a greater than 6-month platinum-free interval were treated using the WTC regimen at the Department of Obstetrics and Gynecology at Kinki University School of Medicine. In this study, we only utilized patients who were first-line failures and platinum-sensitive (platinum-free interval > 6 months) with a minimal survival of 6 months. Fig. 1 shows the patient characteristics of recurrent ovarian cancer. The treatment dose and schedule of WTC consisted of paclitaxel (Taxol, Bristol Meiers) at 60 mg/m^2 for 60 min, and CBDCA (Paraplatin, Bristol Meiers) at AUC = 2 (Carvert's formula) for 60 min, at a weekly administration, for 3 weeks with a 1-week break. The treatment dose was calculated by dividing the

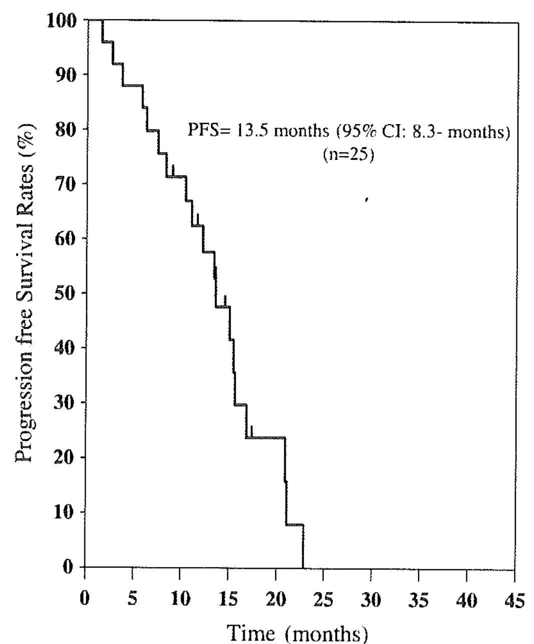

Fig. 1. Progression-free survival of subjected patients. PFS: progression-free survival. Mean follow-up period of patients was 21.9 ± 9.1 months (range: 6–37 months).

standard TC regimen into three (paclitaxel at 180 mg/m^2, CBDCA at AUC = 6). We used premedication with 16 mg of dexamethazone (if patients did not exhibit an allergic reaction, we decreased to 8 mg after the second cycle), 3 mg of granisetron, 50 mg of ranitidine administered intravenously over 60 min, and 50 mg of diphenhydramine per os prior to administration of paclitaxel. G-CSF support was only indicated when patients exhibited an episode of National Cancer Institute Common Toxicity Criteria (NCI-CTC) grade 4 neutropenia or grade 3 neutropenia with neutropenic fever. None of the patients received prophylactic G-CSF supplement. We obtained the full informed consent of participants in the present study including for blood sampling for pharmacokinetic assay. We also obtained permission from the Internal Review Board for this study.

Eligibility criteria and criteria for opening next course

All patients were required to have an over 2-cm diameter of measurable disease by computed tomography (CT) and/or over 75 U/ml of CA-125 elevation (determined on two or more evaluations), and we also referred to magnetic resonance imaging when a patient was judged as having a complete response (CR). Patients also required an Eastern Cooperative Oncology Group (ECOG) performance status of between 0 and 2, and a platinum-free interval of over 6 months. Additional eligibility requirements included a granulocyte count > 2000/mm^3, hemoglobin level > 10.0 g/dl, thrombocyte count > 100,000/mm^3, serum bilirubin level less than 1.5 mg/dl, serum glutamic oxaloacetic transamirase (GOT) and glutamic pyruvic transamirase (GPT) less than 2 × the normal limit, and a serum creatinine concentration < 1.5 mg/dl. Patients with fever over 38°C, cardiac problems requiring clinical treatment, nonepithelial tumor, borderline malignancy, pregnancy, active double cancer, and/or brain metastases were excluded. Moreover, if patient carried over neurotoxicity due to prior chemotherapy, the patient was excluded from this study. We established the following criteria for continuing the next course: granulocyte count > 1500/mm^3, hemoglobin level > 9.0 g/dl, thrombocyte count > 100,000/mm^3, neurotoxicity > grade 2, fever <38°C, and neither progressive disease nor deterioration of complication recognized. If the patients did not satisfy the above criteria for more than 2 weeks or showed progressive disease, they were excluded from further study. Furthermore, if a patient demonstrated partial response (PR), WTC was applied maximum of 18 courses, and no consolidation therapy was applied when patients achieved CR.

Pharmacokinetics

To determine whether paclitaxel satisfies the theoretical antitumor efficacy even when using a low-dose weekly regimen, and to determine whether CBDCA calculated by Calvert's formula satisfies approximate AUC, we performed a pharmacokinetic study to determine the mean AUC and mean peak plasma concentration (C_{max}) of CBDCA and paclitaxel and the mean serum concentration 24 h after administration of paclitaxel in the first treatment course for all eligible patients. Blood samples were taken prior to starting paclitaxel, at the end of administration, and after 30 min, 60 min (start of CBDCA administration), 90 min (30 min after CBDCA administration), 120 min (60 min after CBDCA administration), 3 h (2 h after CBDCA administration), 5 h, 9 h (8 h after CBDCA administration), and 25 h (24 h after CBDCA administration). Ten milliliters of whole-blood samples (5 ml for paclitaxel, 5 ml for CBDCA) were collected in EDTA tubes and immediately centrifuged for 10 min at 3000 rpm, and the plasma was removed. For the study of CBDCA pharmacokinetics, blood samples were centrifuged 15 min at 3000 rpm, using Centrifree (Millipore, Bilerica, MA) to remove the protein. Both samples were stored at −20°C and assayed within 2 weeks by HPLC according to techniques previously described [12].

Patients' evaluation

Responses of patients with measurable disease were determined based on World Health Organization (WHO) guidelines [13]. The CA-125 response criteria by Rustin et al. [14,15] were only applied for patients who did not have measurable disease. The mean CA-125 value of patients without measurable disease was 215.4 ± 45.8 IU/ml (range, 159–289). Toxicity was assessed by NCI-CTC criteria. CT and tumor markers including CA-125 were evaluated every 4 weeks.

Study design

This study was conducted as noncomparative phase II study to evaluate efficacy and toxicity. Moreover, pharmacokinetic study of the paclitaxel and the CBDCA were also applied. Since the response rate for single-agent paclitaxel was 20% [16] to 33% [17] at recurrent disease, this study was designed to be the null hypothesis that the true response probability is less than the clinically significant level of 40% for combination chemotherapy. If it is rejected, we will accept the specified alternative hypothesis that the true response probability is at least a target level of 70% [18,19]. The sample size was calculated as 24 patients, with two-sided, $\alpha = 0.05$, power $(1 - \beta) = 0.9$.

Results

Responses

A total of 25 eligible patients were enrolled in the study, and treated with 451 cycles of WTC regimen. Patient characteristics are shown in Table 1, and the mean follow-up period of patients was 21.9 ± 9.1 months (range; 6–37

Table 1
Characteristics of patients

No. of patients	25
Mean age (range)	58.2 ± 6.8 years (33–71)
Histologic subtype	
Serous	16
Endometrioid	5
Mucinous	2
Clear cell	2
Mean platinum-free interval (range)	11.4 ± 2.7 months (6–18)
Mean treatment courses (range)	18.0 ± 2.8 (9–27)
Prior chemotherapy	
TC	14
CAP	2
CP	9
No. assessable for efficacy	
by measurable disease	18
by CA 125	7
No. assessable for toxicity	25

TC, paclitaxel + carboplatin; CAP, cyclophosphamide + adriamycin + cisplatin; CP, cyclophosphamide + cisplatin.

months). OR determined by WHO criteria for 18 patients with measurable tumor was 84.2% (CR; 9, PR; 7), and OR determined by CA-125 for seven patients was 85.7% (CR; 3, PR; 3). Total OR was 88.0% (CR; 12, PR; 10, Table 2), and progression-free survival was 13.5 months, respectively (Fig. 1).

Toxicity

All patients were assessable for documentation of toxicity. NCI-CTC grade 3/4 for leukopenia and neutropenia was observed in 20% and 36%, respectively, but all patients recovered well, and there were no episodes of neutropenic fever observed. Only one patient exhibited grade 2 anemia, and all patients demonstrated thrombocytopenia of grade 0. Moreover, there were no patients exhibiting grade 3/4 nonhematologic toxicity. Specifically, 56% of the patients did not develop neuropathy. Although all patients developed alopecia, 72% of the patients remained within grade 1 (Table 3). Treatment delay for toxicity of over 7 days was observed in 16.0% of the patients during 1.3% of the treatment cycles. However, there were no patients requiring a treatment delay of over 14 days. G-CSF support was indicated for 12.0% of the patients during 3.3% of the treatment cycles. However, there were no cases requiring blood transfusion, and none of the patients rejected WTC during the treatment course due to subjective symptoms of toxicity (Table 4).

Table 3
Toxicity profile

Adverse events	Grade (no. of cases)					Grade 3/4
	0	1	2	3	4	
Leukopenia	4	4	12	5	0	20%
Neutropenia	3	2	11	7	1	32%
Anemia	10	14	1	0	0	0%
Thrombocytopenia	25	0	0	0	0	0%
Nausea/vomiting	10	1	4	0	0	0%
Neurotoxicity	14	11	0	0	0	0%
Alopecia	0	18	7	–	–	28% (G2)
Renal disorder	24	1	0	0	0	0%
Hepatic disorder	12	10	3	0	0	0%

Toxicity was determined by National Cancer Institute Common Toxicity Criteria.

Pharmacokinetics

The pharmacokinetic study demonstrated that the mean AUC of paclitaxel in the present WTC regimen was 2.74 ± 0.54 μM h, the mean plasma concentration (C_{max}) was 1.36 ± 0.16 μmol/l, and the mean plasma concentration 24 h after administration was 0.023 ± 0.007 μmol/l, while the CBDCA AUC and mean C_{max} were 1.92 ± 0.27 μM h and 6.50 ± 1.04 μmol/l, respectively.

Discussion

In treatment for recurrent ovarian cancer, efficacy for survival as well as acceptability of the regimen among patients should be considered because most recurrent diseases are not considered curable. Therefore, it is important to be concerned with treatment benefit as determined by a comparison between direct effects and adverse effects. Although WTC is a safe and useful regimen, the optimal approximate dose of paclitaxel and

Table 2
Clinical responses

Cases	No. of cases	Response		
		CR	PR	OR (95% CI)
Patients with assessable disease (determined by WHO criteria)	18	9	7	84.2% (0.65–0.98)
Patients without assessable disease (determined by CA-125)	7	3	3	85.7% (0.42–0.99)
Total	25	12	10	88.0% (0.68–0.97)

CR, complete response; PR, partial response; OR, overall response rate.

Table 4
Frequency of unscheduled events

Events	No. of cycles (%)	No. of patients (%)
Total number	451	25
Treatment delay for toxicity		
over 7 days	6 (1.3%)	4 (16.0%)
over 14 days	0 (0.0%)	0 (0.0%)
G-CSF support	15 (3.3%)	3 (12.0%)
Blood transfusion	0 (0.0%)	0 (0.0%)
Rejection of continued treatment	0 (0.0%)	0 (0.0%)

G-CSF, granulocyte colony-stimulating factor.

advantages of weekly partition of CBDCA administration remain unknown. Concerning the administration dose for weekly single-agent paclitaxel treatment, Fennelly et al. [20] conducted a phase I study for relapsed ovarian cancer, and defined the maximum-tolerated dose of weekly paclitaxel as 80 mg/m^2. Moreover, Rosenberg et al. [21] compared single-agent paclitaxel given weekly to the every-three-weeks schedule in patients with previously platinum-treated relapsed ovarian cancer, and reported that weekly paclitaxel at a dose of 67 mg/m^2 showed a better safety profile and seemed to be as effective as an equivalently dosed schedule every 3 weeks. Using a weekly combination treatment with CBDCA, Sehouli et al. [22] conducted a phase I study of first-line chemotherapy for advanced ovarian cancer, and concluded that the recommended dose for a phase II study was paclitaxel at 100 mg/m^2 and CBDCA at AUC = 2. However, clinical studies of WTC for patients with relapsed ovarian cancer have indicated a lower dose of paclitaxel of 80 mg/m^2 [6] or 60 mg/m^2 [7], with consideration to patient acceptability. Generally, WTC was indicated on days 1, 8, and 15 of each 28-day cycle, and the per-week dose intensity of paclitaxel was calculated as 60 mg/m^2/week when using an 80 mg/m^2 weekly administration, and 45 mg/m^2/week when using 60 mg/m^2. These per-week intensities of paclitaxel were similar to those in a previous phase III trials of conventional TC therapy (80 mg/m^2 in weekly regimen is similar to 175 mg/m^2 every 3 weeks in the OV-10 [23] and ICON3 [24] studies. Furthermore, the 60 mg/m^2 weekly regimen is similar to 135 mg/m^2 every 3 weeks, as in the GOG158 [25] and GOG111 [26] studies. However, clinical role of fractionated CBDCA dosing of weekly regimen in recurrent ovarian cancer is still unknown. Moreover, while the ICON4 has revealed that the combination of paclitaxel and CBDCA might offer some advantage over single-agent CBDCA, the advantage of combination therapy over single-agent CBDCA administration followed by single-agent weekly paclitaxel treatment has not been determined. From this point of view, recent clinical trial for lung cancer by Belani et al. [11] was attractive. They reported that combined administration of CBDCA (AUC = 6, Day 1) with the weekly paclitaxel (100 mg/m^2) administration schedule was favorable to the weekly CBDCA (AUC = 2, weekly) and paclitaxel (100 mg/m^2) regimen in overall response rate, mean survival time, and 1-year survival, in a phase II study. Moreover, Rose et al. [Proc Am Soc Clin Oncol 2003, Abstr. 1932] also recently studied the effects of weekly paclitaxel (80 mg/m^2) and every-3-weeks CBDCA (AUC = 5) in platinum-sensitive recurrent ovarian and peritoneal carcinoma, and reported the results as 76% OR, 60% CR rate. Although prior studies, including our present study, of WTC for recurrent ovarian cancer with weekly fractionated CBDCA dosing had started before those reporting on the nonfractionated CBDCA regimen, all of the studies have achieved sufficient results in OR and PFS, as 71.5% OR, 43.0% CR rate [7], a 88% OR, 48% CR rate, and 13.5 months of PFS (present data) when using 60 mg/m^2 of paclitaxel, and 100% OR, 71.0% CR rate, and a 13.7-month median interval until progression [6] and 82.% OR, 53% CR rate, and 11.5 months of PFS when using 80 mg/m^2 of paclitaxel [Dunton CJ et al., Proc Am Soc Clin Oncol 2003, Abstr. 1876]. Even in recent report, Van der Burg et al. [Proc Am Soc Clin Oncol 2004, Abstr. 5058] conducted phase II study of WTC with higher dose of fractionated CBDCA (AUC = 4) regimen for patients with recurrent ovarian cancer, and concluded that the WTC with fractionated CBDCA regimen is highly active in both TC sensitive and TC refractory patients. Therefore, although the rationale of weekly fractionated CBDCA administration has not been established, it was thought that the clinical role of the low-dose WTC with fractionated CBDCA regimen, especially for patients with platinum-sensitive recurrent ovarian cancer, still would not be denied as an optional therapy at this point in time. In the recurrent epithelial ovarian cancer, dose reduction chemotherapy would be considered because recurrent epithelial ovarian cancer is regarded as being chemosensitive com-

Table 5
WTC therapy for relapsed ovarian cancer

Regimen (no. of patients)	Effects			Toxicity			
	OR (%)	CR (%)	PFS	Gr (G3<)	Pl (G3<)	Nr (G2<)	Al (G2<)
T; 80 mg/m^2 C; AUC = 2 (29)[6]	100	71.0	13.7 M	31.0%	31.0%	0.0%	34.5%
T; 80 mg/m^2 C; AUC = 2 (20)[a]	82.0	52.9	11.2 M	20.0%	ND	10.0%	ND
T; 60 mg/m^2 C; AUC = 2 (14)[7]	71.5	43.0	ND	7.1%	0.0%	7.1%	57.1%
T; 60 mg/m^2 C; AUC = 2 (25)[b]	84.2	50.0	13.5 M	32.0%	0.0%	0.0%	28.0%
T; 80 mg/m^2 C; AUC = 5, day 1 (25)[c]	76.0	60.0	ND	64.0%	4.0%	ND	ND
T; 90 mg/m^2 C; AUC = 4 (50)[d]	80.0	24.0	11.0 M	31.0%	9.0%	0.0%	ND

WTC, weekly paclitaxel/carboplatin; OR, overall response rate; CR, complete response rate; PFS, progression-free survival; ND, not described; Gr, granulocytopenia; Pl, thrombocytopenia; Nr, neurotoxicity; Al, alopecia; AUC, area under the concentration curve. Toxicity was determined by the National Cancer Institute Common Toxicity Criteria; G, grade of toxicity.

[a] Dunton CJ et al., Proc Am Soc Clin Oncol 2003, Abstr. 1876.

[b] Our present results.

[c] Rose et al., Proc Am Soc Clin Oncol 2003, Abstr. 1932.

[d] Van der Burg et al., Proc Am Soc Clin Oncol 2004, Abstr. 5058.

pared to lung, stomach, and colorectal cancers. Furthermore, even regarding the weekly paclitaxel dosing in the WTC regimen, Havrilesky et al. [6] reported that 41.4% of the patients required a reduction of the paclitaxel dose to 60 mg/m^2 from 80 mg/m^2 due to neutropenia. Furthermore, Rose et al. [Proc Am Soc Clin Oncol 2003, Abstr. 1932] also recommended paclitaxel at a dose of 60 mg/m^2 in their nonfractionated CBDCA regimen, because 85% of the patients required a paclitaxel dose reduction to 60 mg/m^2 due to treatment toxicities. These results suggest that although the administration schedule of CBDCA was different, 41.4–85% of the patients needed dose reduction to 60 mg/m^2 when 80 mg/m^2 of paclitaxel weekly was indicated. The most resolvable problems of paclitaxel and CBDCA therapy were neurotoxicity and severe alopecia, because most patients rejected the treatment due to those symptoms when we indicated the every-3-weeks regimen (paclitaxel at 175 mg/m^2, CBDCA at AUC = 5). However, in the present study, there were no patients rejecting the WTC regimen, because although 44% of the patients developed neurotoxicity, these symptoms remained grade 1; in addition, 56% of the patients did not develop any neurotoxic symptoms. Although all of the patients developed alopecia, 72% remained grade 1. Moreover, G-CSF support was needed in only 1.3% of the treatment cycles and 12% of the patients, treatment delay of over 7 days was observed only in 3.3% of the treatment cycles and 16% of the patients, and none of the patients required a treatment delay of over 14 days due to toxicity.

A previous in vitro study demonstrated that paclitaxel can promote microtubular assembly and anti-angiogenetic activity even in low doses [27,28], and the approximate serum concentration of paclitaxel that achieved effective growth inhibition of cancer cells while avoiding severe adverse effects was between 0.01 and 0.05 μmol/l. Our pharmacokinetic study indicated that weekly 1-h administration of 60 mg/m^2 paclitaxel achieved the in vitro growth-inhibiting dose even 24 h after administration. Concerning those results, the direct effects between 60 and 80 mg/m^2 of paclitaxel administration in WTC were apparently different, but the contribution of those administered doses to patients' survival was similar (Table 5). Moreover, even compared to the prior studies using nonfractionated paclitaxel and CBDCA regimen, patients' survival was also similar in that OR was ranged as 70% [29], 77% [30], and 91% [31], and PFS was 13 months [29], 10 months [30], and 9 months [31]. Although comparative phase III study of weekly CBDCA and recent collective CBDCA will be needed, considering the possibility of treatment in elderly women and in the treatment of relapsed ovarian cancer and the fact that all patients have the risk of decreased bone-marrow function due to prior chemotherapy, the WTC with weekly CBDCA regimen is still expected to be a useful optional salvage setting. A phase III comparative trial between WTC and every-3-weeks therapy for platinum-sensitive relapsed ovarian cancer and a large phase II trial for primary epithelial ovarian cancer are expected, because WTC shows the potential to achieve therapeutic effects while maintaining patient acceptability.

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Combination effects of irradiation and irinotecan on cervical squamous cell carcinoma cells *in vitro*

TETSUJI TANAKA[1], KAZUNORI YUKAWA[2] and NAOHIKO UMESAKI[1]

Departments of [1]Obstetrics and Gynecology and [2]Physiology, Wakayama Medical University, 811-1 Kimi-idera, Wakayama 641-0012, Japan

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Abstract. Irinotecan HCl (CPT-11) has frequently been used in chemotherapy or concurrent chemoradiotherapy for patients with advanced cervical cancer, although an effective protocol for chemoradiotherapy with CPT-11 has not yet been established. Using the radiosensitive human cervical squamous cell carcinoma cell line ME180 and SN38, a major active metabolite of CPT-11, both the SN38 effects on radiosensitivity and irradiation effects on SN38 sensitivity were investigated to optimize the chemoradiotherapy protocol for CPT-11. SN38 had no effect on radiosensitivity, and irradiation did not affect SN38 sensitivity. Moreover, 3 of 4 post-irradiation surviving subclones obtained from repeatedly irradiated ME180 cells showed no significant changes in their SN38 sensitivities compared with the non-irradiated parent cells. On the other hand, all 7 SN38-resistant subclones established from ME180 cells showed strong reduction in their radiosensitivities. These results suggest that CPT-11 should be administered to advanced cervical cancer patients after, but not before, standard radiotherapy, and concurrent administration of SN38 with radiotherapy should be avoided in order to prevent severe adverse effects, such as watery diarrhea, which is the main adverse effect of pelvic radiotherapy or CPT-11.

Introduction

Patients with unresectable advanced cervical squamous cell carcinoma (SCC) are usually treated with radiotherapy as the therapy of choice, since most cervical SCCs are radiosensitive. However, standard radiotherapy of cervical cancer patients is often non-radical for locally advanced cervical cancer with a huge primary tumor, wide invasion to the pelvic walls, many lymph node metastases, or possible distant micrometastases. Therefore, chemoradiotherapy is sometimes concurrently applied to patients with locally advanced cervical cancer. Cisplatin (CDDP) is one of the most effective anticancer drugs for advanced cervical cancer and has been widely used in concurrent chemoradiotherapy for advanced cervical cancer patients (1-6). When and how CDDP should be injected into cancer patients during radiotherapy has not yet been investigated, although many reports have shown that concurrent chemoradiotherapy with CDDP results in a significantly higher survival ratio than radiotherapy alone (1-5). Tabata *et al* reported that bleomycin, vincristine, mitomycin and cisplatin (BOMP) chemotherapy before radiotherapy in cervical cancer patients resulted in a significantly lower survival ratio than that for patients treated with radiotherapy alone (7). These observations suggest that the radiosensitivity of cancer cells may be reduced by BOMP chemotherapy, and further indicate that chemoradiotherapy of cervical cancer may represent a worse treatment than radiotherapy alone when the chemotherapeutic drugs are inadequately administered.

Recent clinical studies have demonstrated that combination chemotherapies with irinotecan HCl (CPT-11) plus CDDP (8,9) and CPT-11 plus mitomycin C (MMC) (10) produced better therapeutic effects in advanced or relapsed cervical cancer patients than previously used chemotherapies. In Japan, CPT-11 plus MMC is currently one of the first-line chemotherapies for advanced cervical SCC patients. In our preliminary study at Wakayama University Hospital, CPT-11 plus MMC therapy showed 100% efficiency for stage IIIb cervical SCC patients (11). Therefore, the combination of CPT-11 and MMC is the first-line chemotherapy for cervical SCCs at our hospital because patients do not suffer the nausea and vomiting caused by conventional CDDP-based chemotherapy. Since CPT-11 is thought to be a radiosensitizer (12-17), concurrent chemoradiotherapy with CPT-11 has been applied to advanced cancer patients with lung (18-20), rectal (21,22), esophageal (23), and cervical cancer (24), etc. In Japan, a clinical study of concurrent chemoradiotherapy with CPT-11 for patients with advanced cervical cancer has already been initiated. However, there are no reports that indicate when and how CPT-11 should be optimally administered to cervical SCC patients during radiotherapy. In the present study, therefore, radiosensitive human cervical squamous carcinoma cells were used to investigate several options for chemoradiotherapy with CPT-11 to achieve the optimal treatment protocol.

Correspondence to: Dr Tetsuji Tanaka, Department of Obstetrics and Gynecology, Wakayama Medical University, 811-1 Kimi-idera, Wakayama 641-0012, Japan
E-mail: tetanaka@wakayama-med.ac.jp

Key words: CPT-11, irinotecan, chemoradiotherapy, cervical cancer, squamous cell carcinoma, radiosensitivity

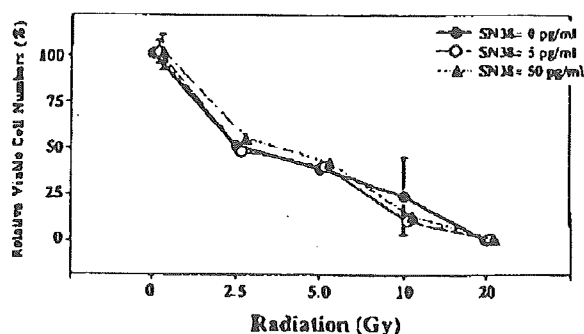

Figure 1. Effects of SN38 on the radiosensitivity of ME180 cells. Within 20 min after the initial addition of SN38 to ME180 cells, the cells were irradiated with various doses of γ-rays. The final SN38 concentrations in the culture media were 0, 5 and 50 pg/ml. The solid line with closed circles shows the control radiosensitivity curve of cells cultured without SN38. The dotted lines with open circles (SN38 =5 pg/ml) and closed triangles (SN38 =50 pg/ml) show the radiosensitivity curves of cells cultured with SN38. There are no significant differences among the 3 radiosensitivity curves.

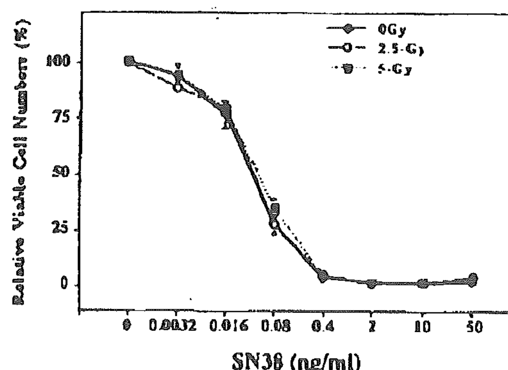

Figure 2. Effects of irradiation on SN38 sensitivity of ME180 cells. Within 20 min after the addition of various concentrations of SN38 to ME180 cells, the cells were irradiated with 2.5 or 5.0 Gy of γ-rays. The solid line with closed circles shows the control SN38 sensitivity curve of cells cultured without irradiation. The dotted lines with open circles (2.5 Gy) and closed squares (5.0 Gy) show the SN38 sensitivity curves of the irradiated cells. There are no significant differences among the 3 sensitivity curves.

Materials and methods

Cell line and cell culture. The radiosensitive human cervical SCC cell line ME180 (25-28) used in this study was obtained from the Japan Resources Cell Bank (JRCB; Tokyo, Japan). All cells used in this study were cultured in Opti-MEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% fetal calf serum (FCS; Equitech Bio Inc., Ingram, TX, USA) and a mixture of 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL). SN38, the major active metabolite of CPT-11 used in this study, was a gift from the Yakult Co. (Tokyo, Japan).

Cell viability assays. The growth-inhibitory effects of radiation and SN38 on ME180 cells were investigated as follows. To examine the effects of radiation, cells in log phase were dispersed with 0.25% trypsin/1 mM EDTA (Gibco-BRL), then cultured overnight in 96-well culture plates (5,000 cells/well). On the second day, the cells were irradiated with various doses of γ-rays using an MBR 1520A irradiator (Hitachi-Medico, Tokyo, Japan). On the fourth day, viable cells were counted using an XTT non-RI colorimetric assay kit (Boehringer Mannheim, Mannheim, Germany). To examine modulatory effects of SN38 on the cell death induced by irradiation, cells were treated with various concentrations of SN38, immediately γ-irradiated at different doses, then cultured for 2 days. Finally, the relative viable cell numbers (expressed as percentages) were calculated using the XTT kit. All experiments were repeated

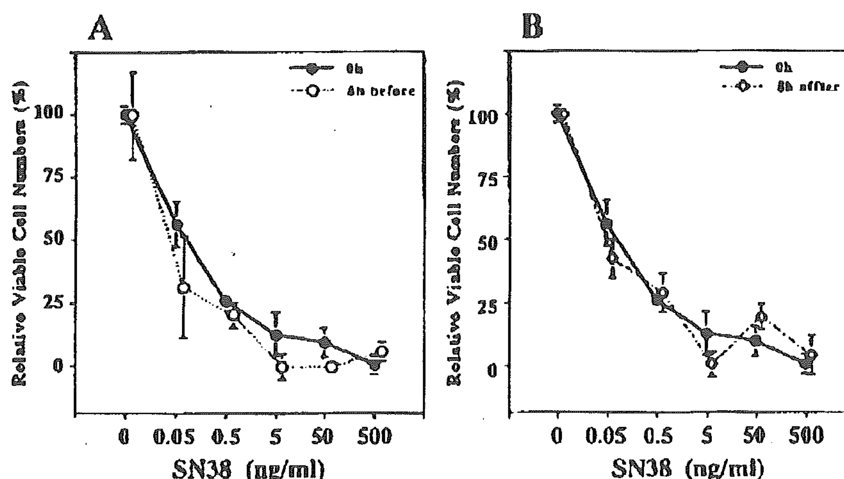

Figure 3. Effects of SN38 treatment and γ-ray irradiation on the SN38 sensitivity of ME180 cells. (A) The solid line with closed circles shows the control SN38 sensitivity curve of cells irradiated with a single dose of 5 Gy immediately after the addition of SN38. The dotted line with open circles shows the SN38 sensitivity curve of cells treated with SN38 at 8 h before irradiation. (B) The solid line with closed circles shows the control SN38 sensitivity curve of cells irradiated with a single dose of 5 Gy immediately after the addition of SN38. The dotted line with open circles shows the SN38 sensitivity curve of cells treated with SN38 at 8 h after irradiation. No significant changes in the SN38 sensitivity curves were observed.

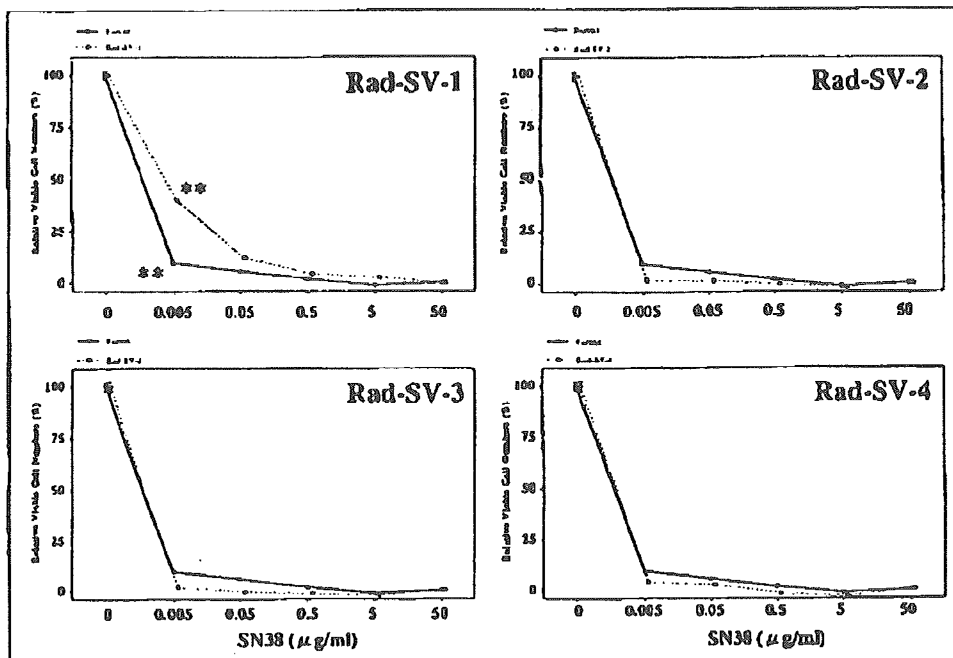

Figure 4. SN38 sensitivities of post-irradiation surviving subclones. The SN38 sensitivities of 4 subclones established from post-irradiation surviving cells were compared with the SN38 sensitivity of non-irradiated ME180 parent cells. The solid lines with closed circles show the control SN38 sensitivity curve of ME180 parent cells. The dotted lines with open circles shows SN38 sensitivity curves of the post-irradiation surviving subclones. Only Rad-SV-1 displays a significantly lower SN38 sensitivity than the non-irradiated parent cells. **$p<0.01$.

2-3 times to verify the results. The data are shown as the mean ± SD, and data comparisons were analyzed using the Student's t-test (n=6) and ANOVA.

Establishment of surviving subclones following repeated irradiation. Post-irradiation surviving subclones were established as follows. ME180 parent cells cultured in a 96-well culture plate (10,000 cells/well) were subjected to 4 consecutive doses of radiation (10 Gy each) once a week, and cultured for about 4 weeks. In a preliminary experiment, more than 90% of the ME180 cells died after a single dose of 10 Gy γ-ray irradiation. Cells collected from the 4 wells containing surviving cancer cell colonies were then sub-cultured at lower cell densities (0.1-20.0 cells/well) using a limiting dilution protocol (29). The cloning efficiencies obtained from the limiting dilution cultures were below 10% (3.7-9.1%). Finally, at 4 months after the initial irradiation, 4 monoclonal post-irradiation surviving subclones, designated Rad-SV-1, Rad-SV-2, Rad-SV-3 and Rad-SV-4, were established.

Establishment of SN38-resistant subclones from ME180 cells. To establish SN38-resistant subclones, ME180 parent cells were cultured with various concentrations of SN38 for 3-5 weeks, and the surviving cells were collected. This collection procedure after SN38 exposure was repeated 4 times. Finally, 7 single cell-derived SN38-resistant subclones, designated SN38r1, SN38r2, SN38r7, SN38rr8, SN38r9, SN38r10 and SN38r12, were established by using the limiting dilution method (29). The monoclonality of each SN38-resistant subclone was confirmed by chromosome analysis (data not shown). The establishment of these SN38-resistant subclones took 11 months (30).

Results

First, the effects of SN38 on the radiosensitivity of ME180 cells were examined. As illustrated in Fig. 1, SN38 did not significantly affect the ME180 radiosensitivity curve. Second, the effects of irradiation on SN38-sensitivity in ME180 were investigated. As shown in Fig. 2, concurrent irradiation did not affect the SN38 sensitivity. Furthermore, irradiation had no effect on the SN38 sensitivity of cells treated with SN38 either before or after irradiation (Fig. 3).

In order to examine the SN38 sensitivities of cells after irradiation, we established 4 post-irradiation surviving subclones and examined their sensitivities to SN38. Three of these subclones showed no significant changes in their SN38 sensitivities compared to the parent ME180 cells, while the remaining subclone (Rad-SV-1) showed a significant reduction in its SN38-sensitivity (Fig. 4).

Finally, the radiosensitivities of SN38-resistant cells were examined to investigate whether radiotherapy would have beneficial effects on cervical cancer patients who relapse after CPT-11-based chemotherapy. As shown in Fig. 5, all 7 SN38-resistant subclone cells were clearly radioresistant.

Discussion

This is the first study to investigate how CPT-11 should be effectively administered to advanced cervical SCC patients treated with radiotherapy. SN38 did not affect radiosensitivity.

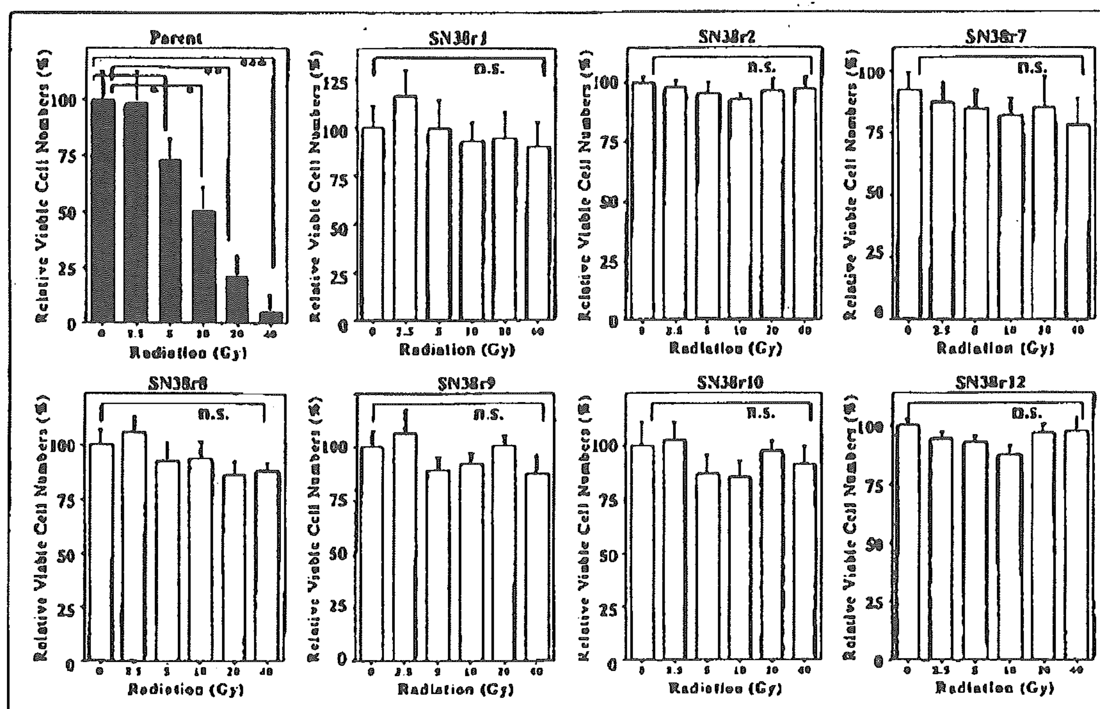

Figure 5. Radiosensitivities of the SN38-resistant cells. The radiosensitivities of 7 SN38-resistant subclones established from ME180 cells were examined. All 7 SN38-resistant subclones (open bars) show clear radioresistance, as 40 Gy of irradiation has no effect on their growth, while almost all parent cells (closed bars) die after irradiation with 40 Gy. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; n.s., not significant.

and irradiation did not affect the SN38 sensitivity of ME180 cells. There were no significant changes in the SN38 sensitivities in 3 of the 4 post-irradiation surviving subclones, whereas all 7 established SN38-resistant subclones were clearly radioresistant. From these results, concurrent chemoradiotherapy with CPT-11 for advanced cervical SCC patients is not thought to have any beneficial effect compared to radiotherapy alone. Watery diarrhea is a well-known adverse effect of CPT-11. Since radiotherapy alone for cervical cancer often causes diarrhea in patients, concurrent chemoradiotherapy with CPT-11 is highly likely to induce severe diarrhea in patients with advanced cervical cancer. In our preliminary clinical trials, many patients suffered severe diarrhea during concurrent chemoradiotherapy with CPT-11. The results of the present study indicate that CPT-11-based chemotherapy may have similar tumor-suppressive effects for cervical cancer after radiotherapy, and radiotherapy may not have a beneficial effect on relapsed patients after CPT-11-based chemotherapy.

The results of the current study reveal that SN38 is not a radiosensitizer for ME180 cells, although many concurrent chemoradiotherapies with CPT-11 have been applied clinically to various types of cancers. To our knowledge, no other *in vitro* studies investigating the effects of radiation and CPT-11 on cervical SCC cells have been reported. The difference between the current results and previous reports (12-17) that CPT-11 is a radiosensitizer may originate from differences in cell lineages or cell lines used in the *in vitro* experiments. However, the ME180 cell line always appears to be responsive to the radiosensitivity-modulatory effects of chemotherapeutic drugs; we have previously examined the combination effects of radiation and other anticancer drugs, such as mitomycin (31), CDDP, docetaxel, pirarubicin (32), doxorubicin, 5-fluorouracil, carboplatin, bleomycin, etc., and found that each of these drugs could enhance or reduce the radiosensitivity of ME180 cells (unpublished data). The present data reveal that SN38 did not enhance the radiosensitivity of ME180 cells, and radiation did not enhance the SN38 sensitivity of ME180 cells. These results indicate that concurrent chemoradiotherapy with CPT-11 for cervical SCCs may have an additive, but not a synergistic cytotoxic effect. In our preliminary clinical experiences with concurrent chemoradiotherapy with CPT-11 for cervical cancer patients, the chemoradiotherapy frequently induced severe watery diarrhea and grade IV myelosuppression in patients, and did not appear to prolong the survival time or time to progression-free survival of patients. In conclusion, treatment protocols for cervical cancer patients involving chemoradiotherapy with CPT-11 need to be further investigated and discussed.

Acknowledgements

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Radiation-induced cell death is independent of the apoptotic signals mediated by death-associated protein kinase in human cervical squamous cell carcinoma cells

TETSUJI TANAKA[1], TAO BAI[1], KAZUNORI YUKAWA[2] and NAOHIKO UMESAKI[1]

Departments of [1]Obstetrics and Gynecology and [2]Physiology, Wakayama Medical University, 811-1 Kimi-idera, Wakayama 641-0012, Japan

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Abstract. Death-associated protein kinase (DAPK) was originally identified as a positive mediator of interferon-γ (IFNγ)-induced apoptosis in cervical cancer cells, and interferons have been reported to enhance radiosensitivity in various types of squamous cell carcinoma. To examine whether DAPK can regulate cancer cell radiosensitivity, we investigated DAPK expression and radiosensitivity in human cancer cell lines, including the cervical squamous cell carcinoma cell line, ME180, which is both radiosensitive and IFNγ-sensitive. Of the 5 human cancer cell lines examined, ME180 cells were the most radiosensitive, but their level of DAPK protein expression was undetectable by Western blotting. A comparative study of ME180 cells with 2 other uterine cancer cell lines, HHUA and HOKUG, revealed no significant relationships between cellular radiosensitivity and DAPK protein expression or hypermethylation of the DAPK promoter CpG island. INFγ dose-dependently inhibited ME180 cell proliferation, but did not induce any cell death. IFNγ significantly enhanced the radiosensitivity of ME180 cells with a slight increase in DAPK protein expression, while irradiation significantly reduced their sensitivity to the growth-inhibitory signals of INFγ. Analyses of 6 monoclonal cisplatin-resistant subclones established from ME180 cells revealed that all 6 were significantly more radioresistant than the parent ME180 cells without any change in the DAPK protein expression. These results indicate that DAPK does not regulate radiation-induced cell death and that it cannot be either a target molecule for radiotherapy with gene therapy or a prognostic marker for cervical cancer patients treated with radiotherapy.

Correspondence to: Dr Tetsuji Tanaka, Department of Obstetrics and Gynecology, Wakayama Medical University, 811-1 Kimi-idera, Wakayama 641-0012, Japan
E-mail: tetanaka@wakayama-med.ac.jp

Key words: death-associated protein kinase, interferon-γ, radiosensitivity, squamous cell carcinoma, cervical cancer

Introduction

Death-associated protein kinase (DAPK) is a 160-kDa cytoskeleton-associated calcium/calmodulin-dependent serine/threonine kinase that was initially identified as a positive mediator of interferon-γ (IFNγ)-induced programmed cell death in HeLa cells, a human cervical adenocarcinoma cell line (1). In addition, DAPK is also involved in the various types of apoptosis induced by tumor necrosis factor-α, Fas (2), c-myc, E2F (3) and detachment from the extracellular matrix (4). Furthermore, the establishment of experimental metastases in syngeneic mice suggested a unique mechanism, linking the suppression of apoptosis with the formation of lung metastases through the loss of DAPK expression (4). The DAPK protein expression level is thought to play an important role in the IFNγ-induced apoptosis of HeLa cells, since the cell death was inhibited by suppression of DAPK expression (1). Interferons have been reported to enhance radiosensitivity in various types of cancer cells (5-14). This finding and the observation that DAPK mediates IFNγ-stimulated apoptosis in HeLa cells suggest there may be a relationship between DAPK-mediated signals and the regulation of radiosensitivity. However, no studies have investigated the interactions between DAPK-mediated signals and radiation-induced cell death. If DAPK-mediated signals are associated with radiation-induced cell death. DAPK may be useful as a candidate target for gene therapy for cancer patients during radiotherapy. DAPK may also be a clinical marker for predicting the radiosensitivity of tumors. Furthermore, dysregulated DAPK expression may be a cause of acquired radioresistance in tumor cells. Since patients with advanced cervical squamous cell cancer are often treated with radiotherapy, a positive relationship between DAPK signaling and radiation-induced cell death signals may lead to a new therapeutic application to cancer therapy. However, since aberrant methylation of the CpG island in the DAPK promoter region frequently occurs in a variety of human primary squamous cell carcinomas, including skin cancer (15), head and neck cancer (16,17), lung carcinoma (18-20) and oral cancer (21), as well as uterine cervical carcinoma (22,23), associations between DAPK-mediated signals and radiosensitivity in cervical squamous cancer cells may be rare. However, if radiation could induce DAPK expression in

cancer cells, the induced DAPK-mediated apoptotic signals may stimulate radiation-induced cell death. This hypothesis has never previously been examined. In the current study, we therefore investigated the possibility of a direct relationship between DAPK expression and radiosensitivity in human uterine cancer cell lines, whose cell proliferation can be inhibited by IFNγ, as well as in HeLa cells.

Materials and methods

Cell lines and culture. The THP-1 (human myeloid leukemia) (24), ME180 (human cervical squamous cell carcinoma) (8,12,14) and HeLa (human cervical adenocarcinoma) (1) cell lines were purchased from the JCRB Cell Bank (Japan Collection of Research Bioresources Cell Bank, Tokyo, Japan). The HHUA (human highly-differentiated endometrial adenocarcinoma) (25) and HOKUG (human cervical glassy cell carcinoma) (26) cell lines were obtained from the Riken Cell Bank (Tsukuba, Japan). All cells were cultured in OPTI-MEM medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FCS; Equitech Bio Inc., Ingram, TX, USA), penicillin (PC) (100 U/ml), streptomycin (SM) (100 μg/ml) and fungizone (0.25 μg/ml; Invitrogen) in 5% CO_2/95% air at 37˚C.

Cell viability assay. The growth-inhibitory effects of radiation or IFNγ on the cell lines were investigated as follows. To investigate the effect of radiation, cells in the log-phase were initially dispersed with 0.25% trypsin/1 mM EDTA (Invitrogen Corp.) and subsequently cultured overnight in 96-well culture plates (5,000 cells/well). On day 2, the cells were irradiated with various doses of γ-rays using an MBR 1520A irradiator (Hitachi-Medico, Tokyo, Japan). On day 4, the viable cells were counted using an XTT non-RI colorimetric assay kit (Boehringer Mannheim, Mannheim, Germany). To examine the modulatory effects of IFNγ on the cell death induced by irradiation, cells were treated with various concentrations of recombinant human IFNγ (courtesy of Shionogi & Co. Ltd., Osaka, Japan), immediately γ-irradiated at different doses and then cultured for 2 days. Finally, the relative viable cell numbers (expressed as percentages) were calculated using the XTT kit. All experiments were repeated 2-3 times to verify the results. The data are shown as the mean ± SD, and data comparisons were analyzed by Student's t-test (n=6) and ANOVA.

Western blot analysis of DAPK expression. After reaching confluency, the cell lines were washed 3 times with ice-cold phosphate-buffered saline (PBS), lysed with 0.5 ml of sample buffer (250 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 2% 2-mercaptoethanol), transferred to a microcentrifuge tube and boiled for 5 min. The protein concentrations were determined from the UV absorption at 280 nm. Aliquots of each cell lysate containing 200 μg of protein were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane (ATTO Corp., Tokyo, Japan) using a semi-dry electroblotting apparatus (ATTO Corp.) at 2 mA/cm² for 45 min. The membrane was blocked with 5% skimmed milk for 1 h at room temperature and subsequently incubated overnight at 4˚C with a mouse monoclonal anti-human DAPK antibody (clone 55; Sigma, St. Louis, MO, USA) that also recognizes murine DAPK. A mouse monoclonal anti-human actin antibody (Sigma) was used to detect actin as an internal protein control. After stringent washing with TBS-T (20 mM Tris, pH 7.6, 0.5 M NaCl, 0.1% Tween-20), the membrane was incubated with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma) for 1 h at room temperature. The membrane was further washed with TBS-T, developed with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and then scanned with a luminocapture (type AE6955; ATTO Corp.).

Genomic DNA extraction and bisulfite modification for DAPK-methylation-specific polymerase chain reaction (DAPK-MS-PCR). Genomic DNA was isolated from the cell lines using a SepaGene kit (Sanko-Junyaku Ltd., Tokyo, Japan) according to the manufacturer's instructions. The DNA concentration was determined from the UV absorptions at 260 nm and 280 nm. Genomic DNA was modified by chemical treatment with sodium bisulfite (Sigma) as described previously (27,28). In this reaction, all unmethylated cytosines were converted to uracils, while the methylated cytosines remained unaltered. Briefly, 2 μg/50 μl of DNA was denatured by adding freshly prepared sodium hydroxide to a final concentration of 0.3 M, and incubating the mixture for 20 min at 37˚C. Next, 30 μl of freshly prepared 10 mM hydroquinine (Sigma) and 520 μl of 3 M sodium bisulfite (pH 5.0) were added to the DNA solutions, mixed and incubated for 16 h at 55˚C. The DNA samples were then desalted using the Wizard DNA Clean-Up System (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol, redissolved in 50 μl of autoclaved distilled water, desulfonated with 0.3 M NaOH for 20 min at 37˚C and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 20 μl of autoclaved distilled water and either used immediately or stored at -70˚C.

DAPK-MS-PCR. DAPK-MS-PCR was performed as described previously (29). Briefly, the bisulfite-modified DNA was used as a template for stage I PCR amplification to generate a 209-bp fragment of the DAPK gene that included a portion of its CpG-rich promoter region. The stage I PCR primers recognized the modified DNA but could not discriminate between methylated and unmethylated alleles. The sequences of the stage I primers were: DAPK forward, 5'-GGTTGTTT CGGAGTGTGAGGAG-3'; and DAPK reverse, 5'-GCTATC GAAAACCGACCATAAAC-3'. Hotstart Taq™ DNA polymerase (Qiagen Ltd., Germany) in a 25 μl volume was used for all PCR amplifications. The stage I PCR amplification was carried out as follows: 95˚C for 15 min, then 35 cycles of 94˚C for 1 min for denaturation, 58˚C for 150 sec for annealing and 72˚C for 150 sec for extension, followed by a final extension at 72˚C for 10 min.

The stage I PCR products were diluted 50-fold, and 5 μl was subjected to the stage II PCR amplification using primers specifically designed for methylated or unmethylated DNA in the promoter region of the DAPK gene.

The sequences of the primers used to selectively amplify the unmethylated and methylated alleles of the DAPK gene during stage II amplification were as follows: unmethylated

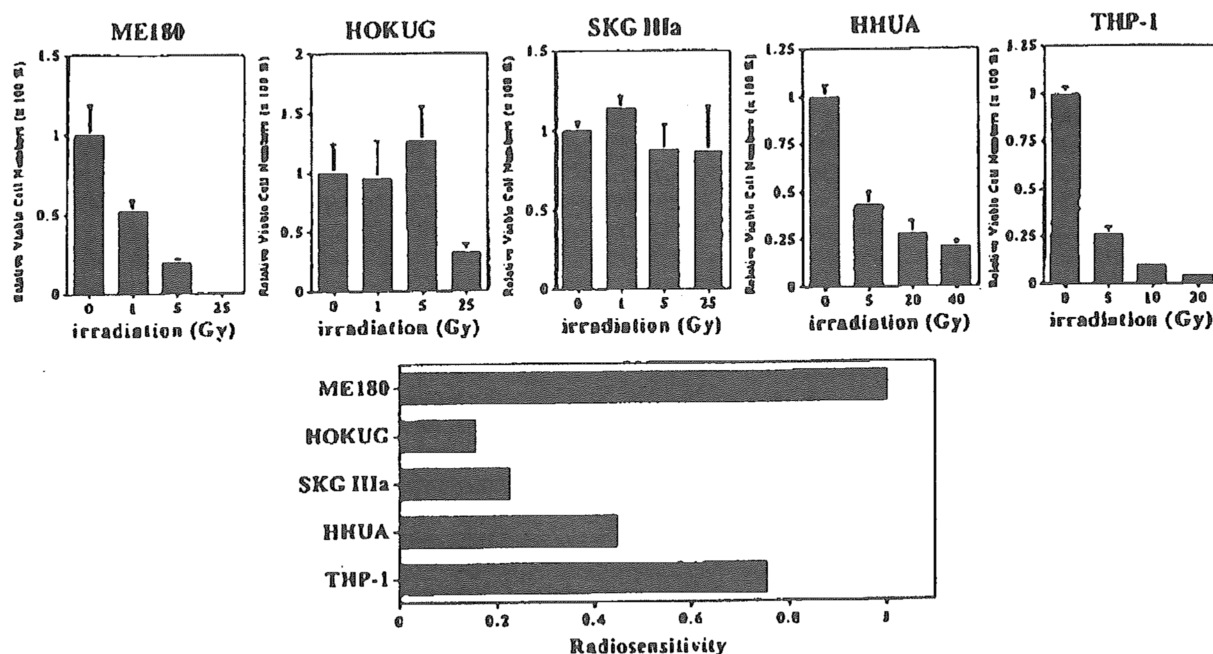

Figure 1. Radiosensitivity tests of 5 human cancer cell lines. The upper figures show the relative cell viabilities (%) of 5 human cancer cell lines. Radiosensitivity was determined as a mean viability ratio against ME180 cells (radiosensitivity = 1), i.e the mean viability (%) of 5-Gy irradiated ME180 cells per mean viability (%) of 5-Gy-irradiated cancer cell.

DNA forward, 5'-GGAGGATAGTTGGATTGAGTTAATGTT-3'; unmethylated DNA reverse, 5'-CAAATCCCTCCCAAACACCAA-3'; methylated DNA forward, 5'-ATAGTCGGATCGAGTTAACGTC-3'; and methylated DNA reverse, 5'-AAAACTAACCGAAACGACGACG-3'. The annealing temperature was increased to 65°C and the annealing time was reduced to 90 sec for 40 cycles. The stage II PCR amplified 153 and 106 bp products from methylated and unmethylated DAPK genes, respectively.

Finally, these PCR products were electrophoresed in a 2.0% agarose gel at 50 V for about 1 h, and then visualized by staining with 5 μg/ml ethidium bromide.

DNA degradation assay using agarose gel electrophoresis. ME180 cells in the log phase were detached with 0.25% trypsin/1 mM EDTA, and then cultured overnight in culture dishes (3×10^6 cells/dish) containing OPTI-MEM/5% FCS/PC/SM. On day 2, IFNγ (200 IU/ml) was added to the cells, followed by irradiation with 3.2-Gy of γ-ray. On day 4, genomic DNA was extracted from all cells, including the dead ones, using a SepaGene DNA extraction kit (Sankyo-Junyaku Co. Ltd.) and treated with 100 μg/ml of RNase A (Sigma) in TE (10 mM Tris, pH 8.0, 2 mM EDTA) for 90 min at 37°C to remove any RNA contamination. Approximately 20 μg of the genomic DNA from 5×10^5 cells was then electrophoresed in a 1.4% agarose gel at 50 V for about 2 h, stained with 5 μg/ml of ethidium bromide and visualized by UV fluorescence.

Establishment of cisplatin (CDDP)-resistant subclones from ME180 cells. To establish CDDP-resistant subclones, ME180 cells were cultured with

weeks and the surviving cells were collected. This collection procedure after CDDP exposure was repeated 4 times. Finally, 6 single cell-derived CDDP-resistant subclones, designated CDDPr1, CDDPr2, CDDPr3, CDDPr4, CDDPr5 and CDDPr6, were established by the limiting dilution method (30). The monoclonality of each CDDP-resistant subclone was confirmed by chromosome analysis (data not shown). The establishment of these CDDP-resistant subclones took 1 year.

Results

Radiosensitivity tests of the 5 human cancer cell lines revealed that ME180 cells were the most radiosensitive (Fig. 1). The results also showed moderate radiosensitivity in HHUA cells and low radiosensitivity in HOKUG cells.

The effects of IFNγ on cell proliferation were examined because DAPK was initially cloned to stimulate IFNγ-induced apoptosis in HeLa cells. IFNγ dose-dependently inhibited the proliferation of both ME180 and HHUA cells (Fig. 2).

Next, the effect of IFNγ on the radiosensitivity of ME180 cells was examined. As shown in Fig. 3A, INFγ significantly enhanced the radiosensitivity. A DNA degradation assay revealed a small enhancement of DNA degradation in ME180 cells treated with radiation and IFNγ, but no apoptosis-specific DNA fragmentation was detected (Fig. 3B). Cells treated with IFNγ alone did not show any DNA degradation or fragmentation, suggesting that the inhibition of ME180 cell growth by IFNγ in Fig. 2A is without apoptosis. In HHUA