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*

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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.

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

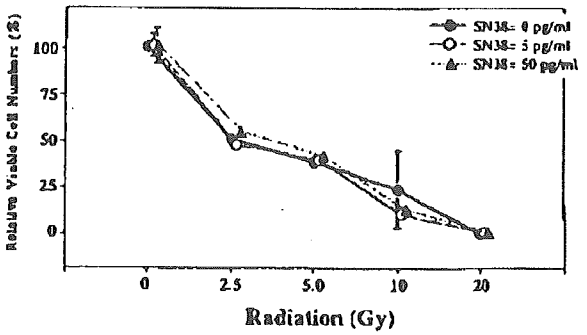

Figure 1. Effects of SN38 on the radiosensitivity of ME180 cells. Within 20 min after the initial addition of SN38 to ME180 cells, the cells were irradiated with various doses of γ-rays. The final SN38 concentrations in the culture media were 0, 5 and 50 pg/ml. The solid line with closed circles shows 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.

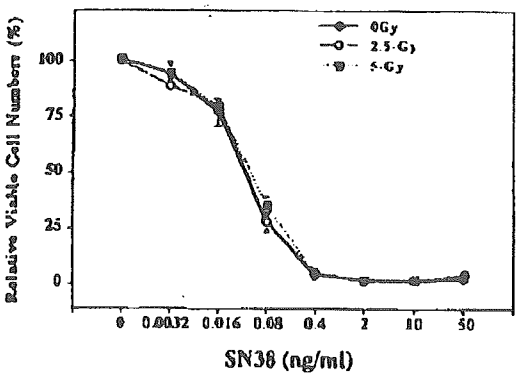

Figure 2. Effects of irradiation on SN38 sensitivity of ME180 cells. Within 20 min after the addition of various concentrations of SN38 to ME180 cells, the cells were irradiated with 2.5 or 5.0 Gy of γ-rays. The solid line with closed circles shows the control SN38 sensitivity curve of cells cultured 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

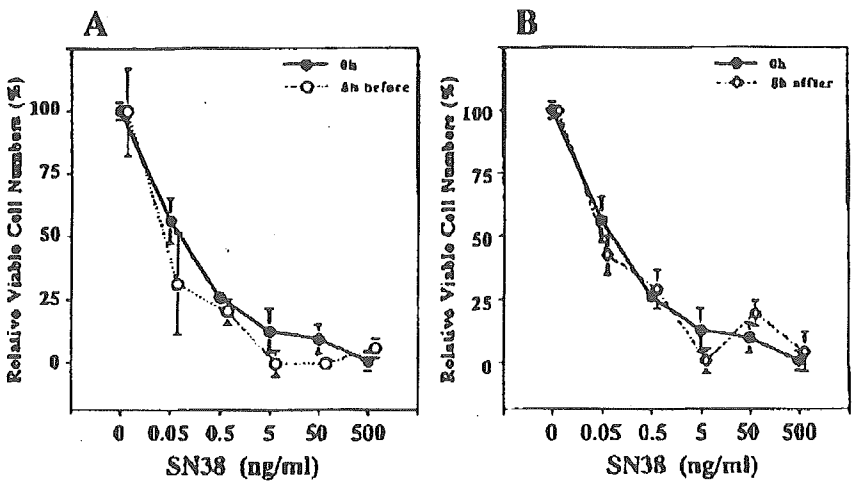

Figure 3. Effects of SN38 treatment and γ-ray irradiation on the SN38 sensitivity of ME180 cells. (A) The solid line with closed circles shows the control SN38 sensitivity curve of cells irradiated with a single dose of 5 Gy immediately after the addition of SN38. The dotted line with open circles shows 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.

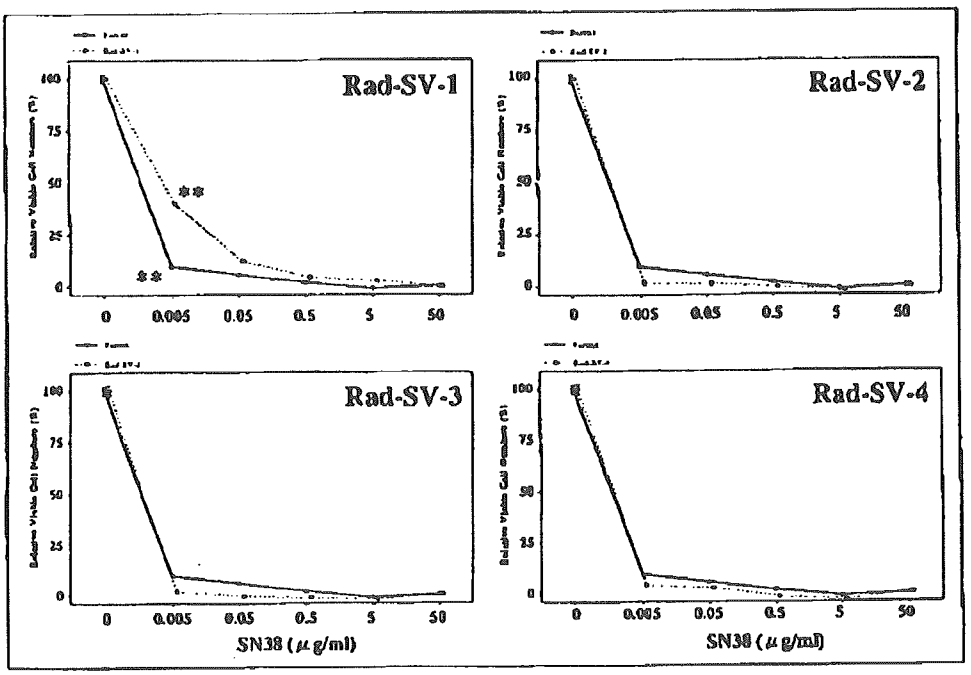

Figure 4. SN38 sensitivities of post-irradiation surviving subclones. The SN38 sensitivities of 4 subclones established from post-irradiation surviving cells were compared with the SN38 sensitivity of non-irradiated ME180 parent cells. The solid lines with closed circles show the control SN38 sensitivity 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, SN38r8, 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.

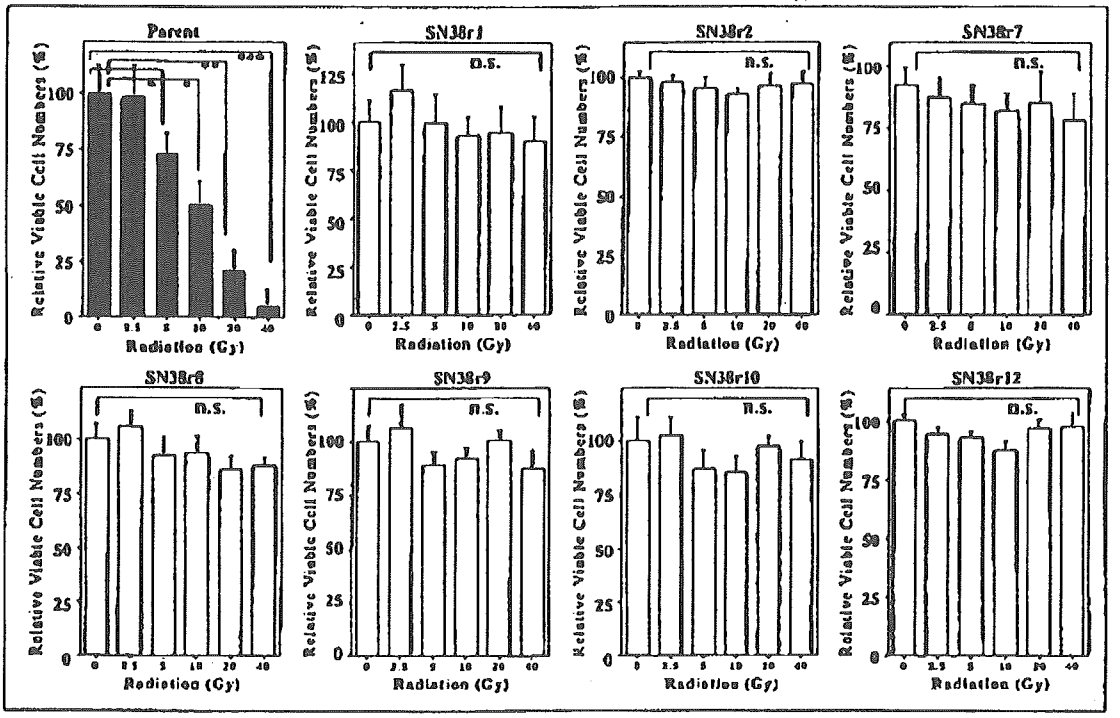

Figure 5. Radiosensitivities of the SN38-resistant cells. The radiosensitivities of 7 SN38-resistant subclones established from ME180 cells were examined. All 7 SN38-resistant subclones (open bars) show clear radioresistance, as 40 Gy of irradiation has no effect on their growth, while almost all parent 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

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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.

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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

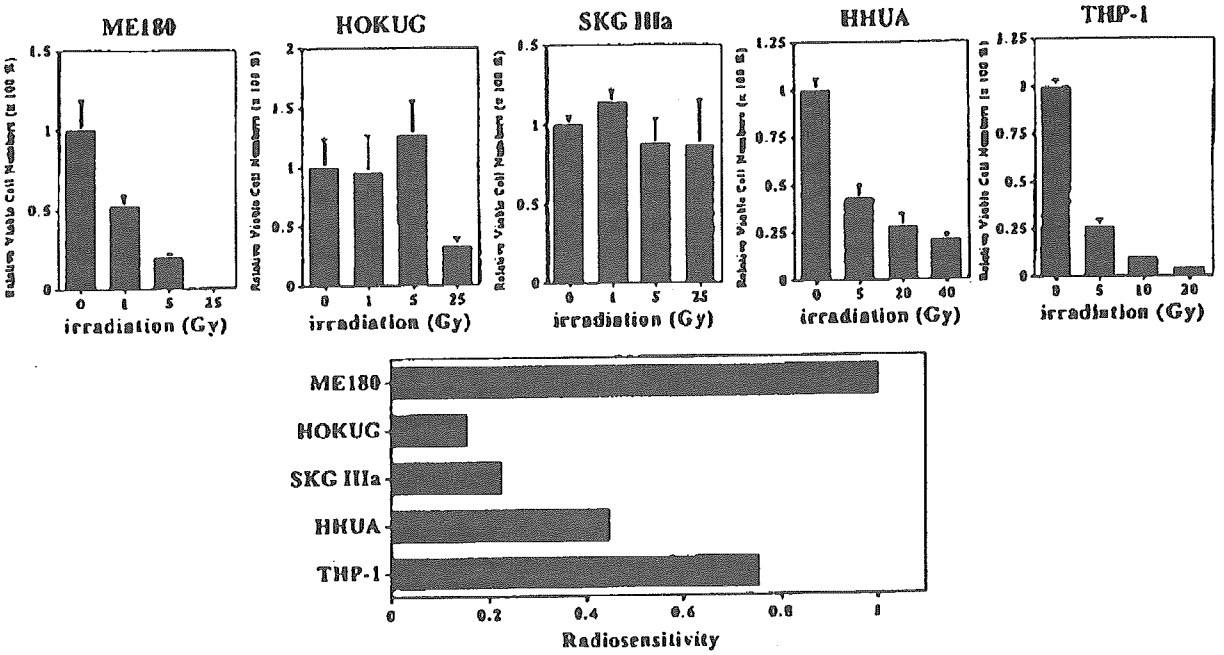

Figure 1. Radiosensitivity tests of 5 human cancer cell lines. The upper figures show the relative cell viabilities (%) of 5 human cancer cell lines. Radiosensitivity was determined as a mean viability ratio against ME180 cells (radiosensitivity = 1), i.e the mean viability (%) of 5-Gy irradiated ME180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

DNA forward, 5'-GGAGGATAGTTGGATTGAGTTAAT GTT-3'; unmethylated DNA reverse, 5'-CAAATCCCTCCC AAACACCAA-3'; methylated DNA forward, 5'-ATAGTCG GATCGAGTTAACGTC-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 ($3x10^6$ 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 $5x10^5$ 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 various [illegible] 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

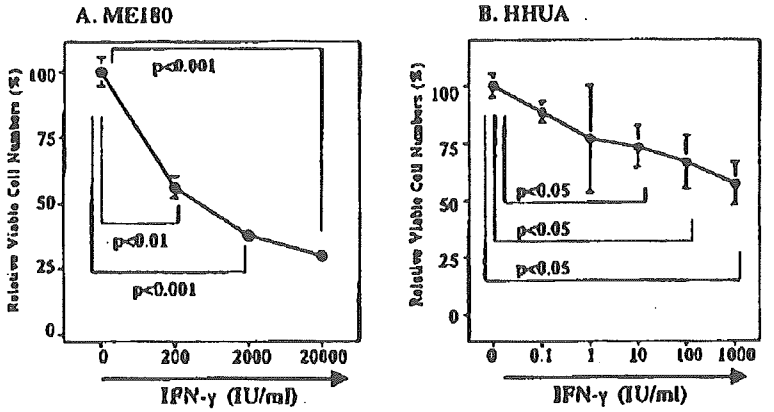

Figure 2. Effects of IFNγ on the proliferation of ME180 and HHUA cells. INFγ dose-dependently inhibits the proliferation of ME180 (A) and HHUA (B) cells.

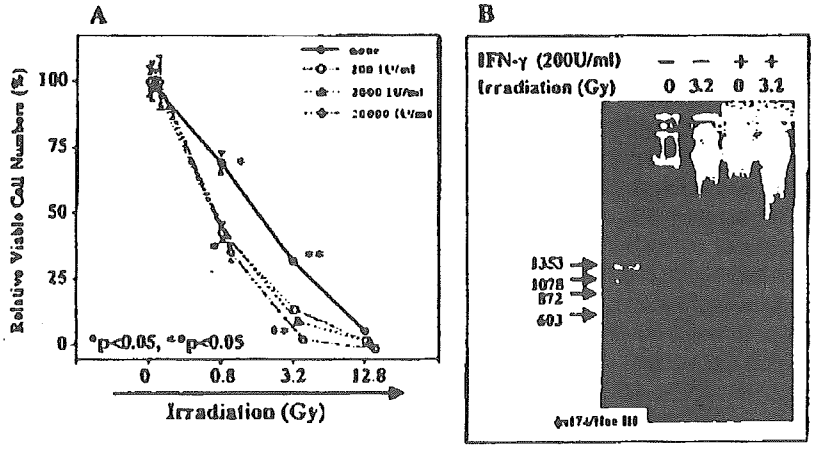

Figure 3. Effects of IFNγ on the radiosensitivity of ME180 cells. A, effects of IFNγ on the radiosensitivity curves of ME180 cells. IFNγ significantly enhances the irradiation-induced growth inhibition of ME180 cells. B, DNA degradation assay. DNA degradation in irradiated ME180 cells is slightly enhanced by IFNγ. No DNA fragmentation is detected.

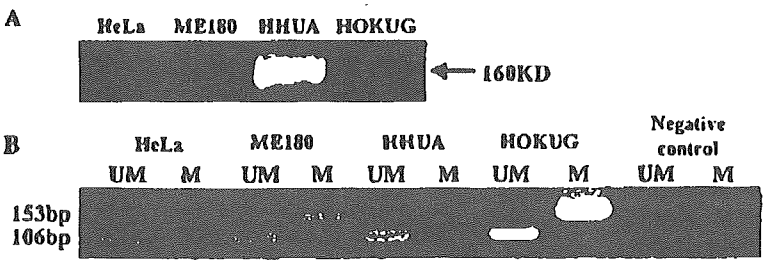

Figure 4. DAPK protein expression and DAPK-MS-PCR in 4 cancer cell lines. A, Western blot analysis of DAPK protein expression in the 4 human uterine carcinoma cell lines. B, DAPK-MS-PCR. The presence of a 153-bp product in lane M indicates the presence of DAPK gene promoter methylation, while a 106-bp product in lane UM indicates the presence of the unmethylated promoter sequence. The DAPK gene promoter is hypermethylated in ME180 and HOKUG cells. Distilled water was loaded in the lanes labeled negative control.

In order to examine whether DAPK expression is involved in the radiosensitivity of cancer cells, Western blot analyses were carried out to detect DAPK protein expression in ME180, HHUA and HOKUG cells. DAPK protein expression was undetectable in both ME180 cells, which were the most radiosensitive, and HOKUG cells, which were the least radiosensitive (Fig. 4A). HHUA cells, which were moderately radiosensitive, showed strong DAPK expression. To investigate the relationships between DAPK protein expression and CpG island hypermethylation of the DAPK gene promoter regions

Table I. Comparisons of the radiosensitivity and DAPK expression in 3 human uterine cancer cell lines.

	ME180	HHUA	HOKUG
Radiosensitivity	++	+	-
DAPK protein expression	-~±	+++	+
Hypermethylation of the DAPK gene promoter	++	-	++

in the 3 cell lines as well as in HeLa cells, MS-PCR for DAPK was performed. Methylation of the CpG islands was detected in ME180 and HOKUG cells, but not in HeLa or HHUA cells (Fig. 4B). These results indicate that the DAPK expression in these 4 cell lines was regulated by methylation of the DAPK CpG island. Taking all these results together, there was no relationship between radiosensitivity and DAPK protein expression or DAPK CpG island hypermethylation in the ME180, HHUA and HOKUG cell lines (Table I).

To investigate the relationships between radiation-induced cell death and DAPK-mediated apoptosis, changes in the DAPK expression in irradiated ME180 cells were examined. A Western blot analysis revealed that the DAPK protein expression in ME180 cells was slightly, but not significantly, increased after irradiation (Fig. 5A). Considering the result that radiation may induce DAPK expression and the report that DAPK stimulates IFNγ-induced apoptosis in HeLa cells (1), it was hypothesized that irradiation would not reduce the sensitivity of ME180 cells to IFNγ. In fact, however, irradiation significantly reduced the sensitivity of ME180 cells to IFNγ in a dose-dependent manner (Fig. 5B).

INFγ-induced apoptosis in HeLa cells is inhibited by suppression of DAPK expression (1), suggesting that the DAPK expression level plays an important role in IFNγ-induced cell death. In ME180 cells, however, IFNγ inhibited cell proliferation but did not induce cell death (Figs. 2A and 3B). The different effects of IFNγ on growth inhibition and cell death in HeLa and ME180 cells may arise from two independent signals induced by IFNγ, namely growth-inhibitory signals and apoptotic signals. Therefore, CDDP, which inhibits cancer cell growth with the induction of apoptosis, was used to investigate the relationships among CDDP-induced cell death, DAPK protein expression and radiation-induced cell death. We examined DAPK protein expression and radiosensitivity in 6 CDDP-resistant subclones established from ME180 cells in order to investigate the mechanisms of their drug-resistance. As shown in Fig. 6B, all 6 CDDP-resistant subclones were strongly radioresistant. Moreover, Western blot analysis revealed that DAPK protein expression was undetectable in all 6 CDDP-resistant subclones, similar to the case for the parent ME180 cells (Fig. 6C).

Discussion

The results of the present study indicate that the radiosensitivity of cancer cells is highly unlikely to be affected by the DAPK protein expression level. The radiosensitivity of ME180 cells, in which DAPK protein expression is strongly suppressed, is very high, as is usual for cervical squamous cell carcinoma cells. Furthermore, we established 6 CDDP-resistant subclones from ME180 cells that were all much more radioresistant than the parent cells and found that their DAPK expressions were undetectable. These results indicate that the acquisition of CDDP-resistance or radioresistance does not require the induction of DAPK protein expression. From the results of experiments on 3 of the cell lines (summarized in Table I), the radiosensitivity of cancer cells has no relationship with either DAPK protein expression or DAPK CpG island methylation. We strongly suggest that the DAPK protein expression level may have no regulatory function in the radiosensitivity of cancer cells.

The present study has proven that DAPK-mediated signals are not essential for the growth-inhibitory signals of IFNγ. In HeLa cells, IFNγ-induced apoptosis can be inhibited by suppression of DAPK protein expression (1), suggesting that DAPK-mediated signals are essential for the IFNγ-induced apoptosis in these cells. Growth inhibition by IFNγ was found in ME180 cells, in which DAPK protein expression

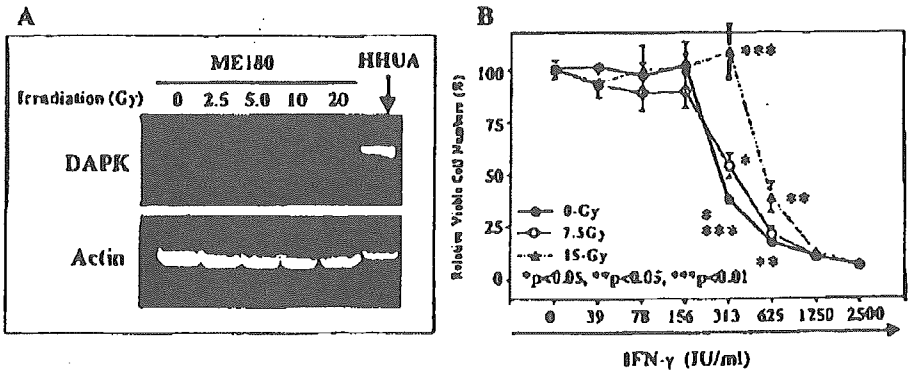

Figure 5. Effects of radiation on DAPK protein expression and sensitivity to IFNγ. A, effects of irradiation on DAPK protein expression in ME180 cells examined by Western blot analysis. DAPK protein expression (normalized to the actin protein expression) is slightly increased after irradiation. B, effects of irradiation on the sensitivity of ME180 cells to IFNγ. Irradiation significantly reduces the IFNγ-sensitivity in a dose-dependent manner.

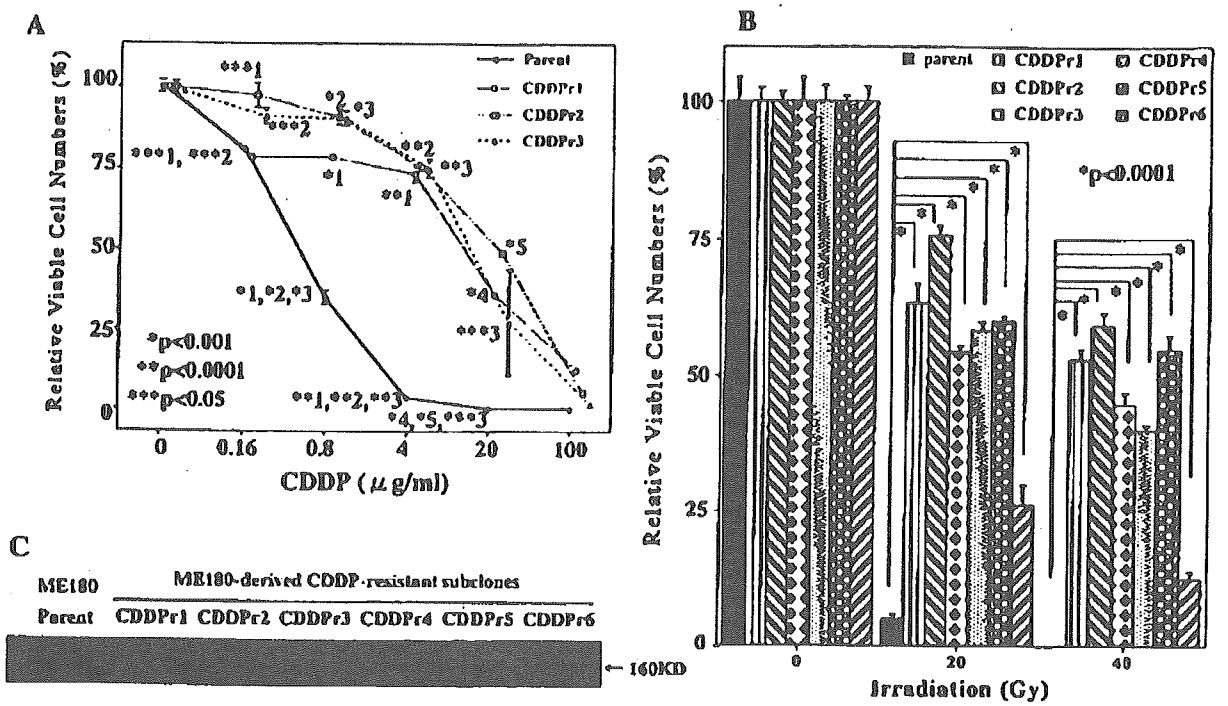

Figure 6. DAPK protein expression and radiosensitivity in ME180-derived CDDP-resistant subclones. A, CDDP-sensitivity of the CDDP-resistant subclones. The CDDP-sensitivity curves of 3 CDDP-resistant subclones, namely CDDPr1, CDDPr2 and CDDPr3, are shown. The other 3 CDDP-resistant subclones showed the same results (data not shown). All the established subclones are significantly more resistant to CDDP than the parent ME180 cells (closed circles with a solid line). Open circles with a dotted line, CDDPr1; open squares with a dotted line, CDDPr2; closed triangles with a dotted line, CDDPr3. B, radiosensitivities of the parent ME180 cells and 6 CDDP-resistant subclones. All 6 CDDP-resistant cells are significantly more radioresistant than the parent cells. C, Western blot analysis of DAPK protein expression in the parent ME180 cells and 6 CDDP-resistant subclones. No DAPK protein expression is detected in any of the 6 CDDP-resistant subclones.

is strongly suppressed. However, although IFNγ strongly inhibited ME180 cell proliferation, it did not induce detectable cell death. In other words, the cell growth inhibition of ME180 cells by IFNγ was not associated with apoptotic changes. These results mean that DAPK-mediated signals are not essential for the growth-inhibitory signals induced by IFNγ-stimulation in ME180 cells. Further studies are required to determine whether or not DAPK-mediated signals are essential for the apoptosis induced by IFNγ, since IFNγ inhibited cell proliferation but did not induce apoptosis in HHUA cells, which show a very high level of DAPK protein expression.

IFNγ enhanced radiation-induced cell death in ME180 cells. On the other hand, radiation significantly reduced the sensitivity to IFNγ, while DAPK protein expression was slightly, but not significantly, enhanced after irradiation. As described above, DAPK-mediated signals are not essential for the growth-inhibitory signals induced by IFNγ in ME180 cells. Therefore, we cannot exclude the possibility that this slight increase in DAPK expression induced by irradiation may enhance radiation-induced cell death. However, since HHUA cells with a high level of DAPK protein expression were much less radioresistant than ME180 cells with an undetectable level of DAPK protein expression, it appears very unlikely that DAPK-mediated signals affect radiation-induced cell death.

In conclusion, the present study has almost proven that there is no relationship between DAPK protein expression level and radiosensitivity in ME180 cells, whose radiosensitivity can be enhanced by IFNγ. From our results, DAPK-mediated signals do not appear to have any effect on radiation-induced cell death although DAPK has been reported to be involved in IFNγ-induced cell death, apoptosis mediated by Fas or TNF-receptor and cell death induced by detachment of cells from the extracellular matrix (1-4). Since DAPK expression in tumor cells is thought to regulate metastasis and prognosis in cancer patients, DAPK has been proposed as a candidate molecular target for gene therapy. However, the current results indicate that DAPK is highly unlikely to be either a molecular target for combined radiotherapy with any gene therapy or a prognostic marker for radiotherapy in cancer patients. Radiation-induced cell death signals are considered to be independent of the DAPK-mediated apoptotic signals.

Acknowledgments

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Radiation enhances cisplatin-sensitivity in human cervical squamous cancer cells *in vitro*

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Summary

Purpose and methods of investigation: Cisplatin (CDDP) is regularly used in concurrent chemoradiotherapy in patients with advanced cervical cancer although an effective protocol of chemoradiotherapy with CDDP has not yet been established. In search of a better chemoradiotherapy protocol, we investigated both CDDP effects on radiosensitivity and irradiation effects on CDDP-sensitivity using the radiosensitive human cervical squamous cell carcinoma cell line ME180.

Results: We found that CDDP did not affect cellular radiosensitivity, and that irradiation significantly enhanced CDDP-sensitivity. Moreover, all the four post-irradiation surviving subclones obtained from repetitively irradiated ME180 cells showed significantly higher CDDP sensitivities than those of the non-irradiated parent cells.

Conclusion: These results suggest that an effective protocol would involve the concurrent administration of CDDP with radiotherapy and further administration following completion of radiotherapy in order to achieve higher CDDP-sensitivities.

Key words: Cisplatin; Chemoradiotherapy; Cervical cancer; Squamous cell carcinoma; Radiosensitivity.

Introduction

Since most cervical squamous cell carcinoma (SCC) cells are radiosensitive, patients with unresectable advanced cervical SCC are usually treated by radiotherapy as the first choice of therapy. However, standard radiotherapy of cervical cancer patients is often non-radical for locally advanced cervical cancers with either huge primary tumors, wide invasion to pelvic walls, many lymph node metastases, or possible distant micrometastases. Therefore, in order to i) eradicate cancer cells outside irradiated fields, ii) enhance the radiosensitivity of cancer cells during radiotherapy, iii) and kill surviving cancer cells after irradiation, chemoradiotherapy has sometimes been concurrently applied to patients with locally advanced cervical cancer.

Cisplatin (CDDP) is thought to be the most effective anticancer drug for cervical cancer. Therefore, CDDP has been the most frequently used worldwide in concurrent chemoradiotherapy in patients with advanced cervical SCC. Several research groups interested in concurrent chemoradiotherapy for cervical cancer patients reported significant increases in survival ratios of cancer patients treated with concurrent chemoradiotherapy using CDDP [1-5]. On the other hand, a few studies could not find any beneficial effects on survival times for patients receiving CDDP chemoradiotherapy [6]. In these studies, usual administration protocols of CDDP in concurrent chemoradiotherapy involved weekly injections of 40-75 mg/m^2 CDDP [1-6]. Recently, a few basic investigations reported the effective concurrent use of CDDP with radiotherapy. In addition, Tabata *et al.* [7] showed that cervical cancer patients pretreated with bleomycin, vincristine, mitomycin and cisplatin (BOMP) chemotherapy including CDDP before radiotherapy demonstrated a lower survival ratio compared with patients treated with radiotherapy alone. These results suggest that the use of BOMP chemotherapy before radiotherapy reduces radiosensitivity of cervical cancer cells. In the present study, we used radiosensitive human cervical squamous carcinoma cells to investigate several options of chemoradiotherapy with CDDP in order to achieve optimal treatment.

Materials and Methods

Cell line and cell culture

In this study, the radiosensitive human cervical SCC cell line ME180 with wild-type p53 genes [8] was used. The ME180 cells were obtained from Japan Resources of 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/100 μg/ml streptomycin (GIBCO-BRL). CDDP used in this study was a gift from Nihon-Kayaku Co. (Tokyo, Japan).

Cell viability assay

Cell proliferation was assessed with the XTT non-RI colorimetric assay kit (Boerhinger Mannheim, Mannheim, Germany). Growth-inhibitory effects of radiation and CDDP on ME180 cells were investigated as follows. Cells in the log phase were initially dispersed with 0.25% trypsin/1 mM EDTA (GIBCO-BRL), and subsequently cultured overnight in 96-well culture plates (5,000 cells/well). On the second day, various doses of γ-rays were used to irradiate the cells using a MBR 1520A irradiator (Hitachi-Medico, Tokyo, Japan). On the fourth day, viable cells were counted with the XTT kit. In order to examine the modulatory effects of CDDP on cell death induced by irradiation, cells were treated with various concentrations of CDDP

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and immediately γ-irradiated at different doses, followed by a 2-day culture. Finally, relative viable cell numbers (expressed as a percentage) were calculated using the XTT kit. All experiments were repeated two or three times to verify the results. Data are reported as means followed by standard deviations (SD), and comparative data (n = 6) were statistically analyzed by ANOVA.

Establishment of surviving subclones following repetitive irradiations

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 four consecutive doses of radiation (10 Gy each) once a week, and cultured for about four weeks. In a preliminary experiment, more than 90% of ME180 cells were killed after a single dose of 10 Gy γ-ray irradiation. Cells were collected from each of the four wells containing surviving cancer cell colonies and sub-cultured with a lower cell density (0.1-20 cells/well) using a limiting dilution protocol. Cloning efficiencies assessed from the limiting dilution cultures were below 10% (3.7%-9.1%). Finally, four months following the initial irradiation, four monoclonal post-irradiation surviving subclones were established.

Results

First of all, effects of CDDP on radiosensitivity of ME180 cells were examined. As illustrated in Figure 1, CDDP did not have any significant effect on ME180 radiosensitivity curves. Secondly, effects of irradiation on ME180 CDDP-sensitivity were investigated and we found that irradiation significantly enhanced CDDP-sensitivity (Figure 2). In order to determine whether post-irradiated cancer cells maintain the higher CDDP-sensitivity following irradiation, we established four post-irradiation surviving subclones according to methods described above and examined their sensitivity to CDDP. All four established subclones demonstrated significant higher CDDP-sensitivities than the parent ME180 cells as shown in Figure 3, suggesting that post-irradiation increases CDDP-sensitivity and this can be maintained for at least four months after initial irradiation.

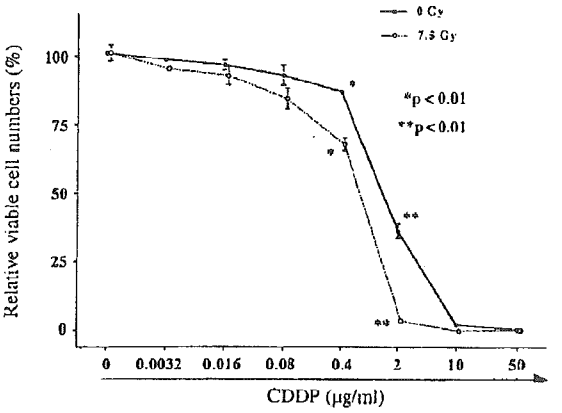

Figure 2. — *Effects of irradiation on CDDP-sensitivity of ME180 cells.*
Within 20 minutes after various concentrations of CDDP were added to ME180 cells, γ-rays were used for radiation. The solid line with closed circles shows the control CDDP-sensitivity curve of cells cultured without irradiation. γ-ray irradiation (7.5 Gy) significantly enhanced the CDDP-sensitivity (dotted line with open circles) (*p < 0.01, **p < 0.01).

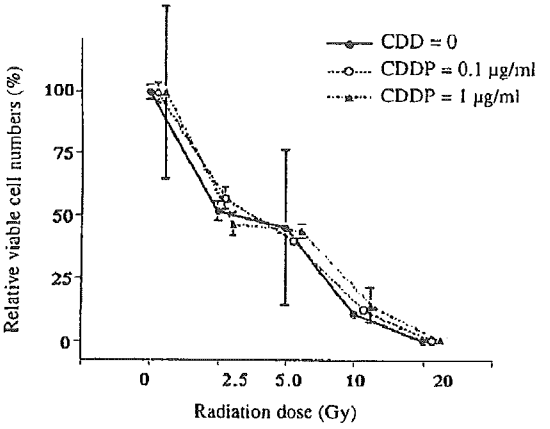

Figure 1. — *Effects of CDDP on radiosensitivity of ME180 cells.*
Within 20 minutes after initial addition of CDDP to ME180 cells, various doses of γ-rays were used for radiation. Final CDDP concentrations in culture media are 0, 0.1, and 1 μg/ml, respectively. The solid lines with closed circles show control radiosensitivity curves of cells cultured without CDDP. The dotted lines with open circles and closed triangles are those of cells cultured with CDDP. There was no significant difference between the three radiosensitivity curves.

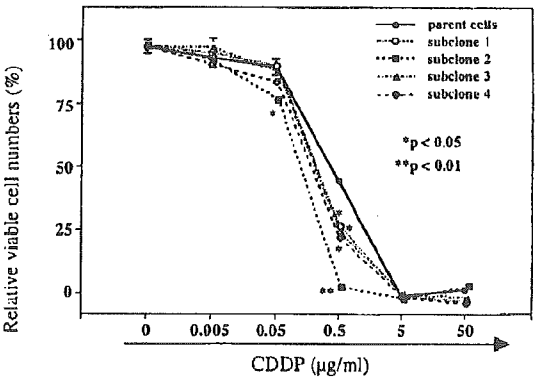

Figure 3. — *CDDP-sensitivity of post-irradiated surviving cells.*
CDDP-sensitivity of the four subclones established from surviving cells following irradiation was compared with the CDDP-sensitivity of non-irradiated ME180 parent cells. The solid line with closed circles shows the control CDDP-sensitivity curve of ME180 parent cells. All four post-irradiated surviving subclones (dotted lines) displayed significantly higher sensitivities to CDDP compared with non-irradiated parent cells (*p < 0.05, **p < 0.01).

Discussion

Radiotherapy is the most commonly used therapy for locally advanced cervical cancers and CDDP is also one of the most effective anticancer drugs used for advanced cervical cancers. Therefore, CDDP has been widely used in concurrent chemoradiotherapy for advanced cervical cancer patients [1-6]. Although many reports showed that concurrent chemoradiotherapy with CDDP resulted in significantly higher survival ratios than those of radiotherapy alone [1-5], there have been few reports on how to effectively administer CDDP during radiotherapy. Studies reporting concurrent chemoradiotherapy with CDDP used weekly injections of 40-75 mg/m^2 CDDP. When and how CDDP should be injected to cancer patients during radiotherapy has not been investigated yet. Tabata et al. reported that BOMP chemotherapy with the use of CDDP before radiotherapy in cervical cancer patients resulted in significantly lower survival ratios than those in patients treated with radiotherapy alone [7]. This report suggests that radiosensitivity of cancer cells may be reduced by BOMP chemotherapy. Additionally these results make us aware that chemoradiotherapy of cervical cancers may provide worse treatment than radiotherapy alone in cases of inadequate administration of chemotherapeutic drugs.

In our study, we used radiosensitive human cervical SCC cells to report optimal treatment conditions for combined chemoradiotherapy with CDDP. Although CDDP does not affect radiosensitivity of cancer cells, irradiation significantly enhances CDDP-sensitivity. Moreover, we found that surviving cancer cells following irradiation have a higher CDDP-sensitivity. These results suggest that CDDP should be administered to cervical cancer patients not before but after irradiation. Our results coincide with clinical results reported by Tabata *et al.* [7]. In conclusion, post-irradiation CDDP injection may be a better treatment than concurrent chemoradiotherapy alone because irradiated cancer cells may retain higher CDDP-sensitivity for several months after irradiation. Moreover, our proposed investigative procedures can be applied to other studies aimed to optimize other combinations of anticancer drugs with radiotherapy in cervical cancer patients.

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