Distant metastases developed in 25 patients. The most frequent site was the paraaortic lymph node (n = 13), followed by Virchow's node (n = 8), liver (n = 5), bone (n = 5), and lung metastasis (n = 3). Ten patients experienced both locoregional and distant failure.

Univariate Analysis

Survival

Univariate analyses for each outcome are summarized in Table 4.

The tumor size/volume derived from MRI showed no significant influence on either OAS or DFS. Patients with larger calculated volumes greater than 100 ml had a tendency toward poorer OAS (p = 0.096) and DFS (p = 0.072). Patients with disease of D_l greater than 60 mm had a tendency toward lower DFS than those with D_l less than or equal to 60 mm (p = 0.082). A positive lymph node judged by MRI proved to be a significantly unfavorable factor for DFS (p = 0.0247, Fig. 1). Patients with bilateral disease fixation had a significantly poorer OAS than those with unilateral disease (p = 0.0001). The same correlation was also noted as a function of DFS (p = 0.0332, Fig. 2). Patient age less than or equal to 50 years proved to be a significantly adverse factor for both OAS ($p < 0.0001$) and DFS ($p < 00001$). We found no statistically significant difference between the outcomes grouped by substage (IIIA vs. IIIB), OTT, tumor marker of SCC-antigen, and hydronephrosis.

Pelvic Control

Tumor size/volume showed no apparent influence on PC in this analysis. Presence or absence of a pelvic lymph

TABLE 4. Univariate analyses of the clinical outcome according to predictive variables; 5-year rate calculated by the Kaplan-Meier method

Predictive variable	Patient number	OAS	DFS	PC	DMFS
Tumor diameters Anteroposterior (D_{ap})					
≤60 mm	74	61.2	50.0	73.5	68.3
>60 mm	6	44.4	33.3	83.3	33.3
		p = 0.61	p = 0.19	p = 0.74	p = 0.01
Lateral (D_l)					
≤60 mm	71	62.3	50.6	73.8	69.7
>60 mm	9	41.7	33.3	77.8	33.3
		p = 0.25	p = 0.08	p = 0.98	p = 0.002
Craniocaudal (D_{cc})					
≤60 mm	65	60.7	49.8	71.8	70.5
>60 mm	15	55.9	45.7	86.7	45.7
		p = 0.50	p = 0.42	p = 0.40	p = 0.02
Maximum (D_{max})					
≤60 mm	62	60.7	48.6	70.3	70.4
>60 mm	18	55.0	49.4	88.9	49.4
		p = 0.69	p = 0.64	p = 0.21	p = 0.03
Tumor volume					
≤100 cm^3	69	63.4	51.3	73.4	70.7
>100 cm^3	11	32.3	34.1	81.8	34.1
		p = 0.10	p = 0.07	p = 0.79	p = 0.0006
Lymph node					
Negative	52	61.8	53.7	76.0	71.8
Positive	28	52.8	36.4	69.3	50.4
		p = 0.19	p = 0.02	p = 0.41	p = 0.03
Pelvic fixation*					
Bilateral	16	37.5	37.5	67.7	46.2
Unilateral	50	75.7	59.0	83.1	75.5
		p = 0.001	p = 0.03	p = 0.11	p = 0.02
Patient age (yrs)					
≤50	7	14.3 (3Y)	0 (15M)	14.3 (3Y)	0 (15M)
>50	73	64.0	53.4	80.0	68.7
		$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	p = 0.004

DFS, disease-free survival; DMFS, distant metastasis-free survival; OAS, overall survival; PC, pelvic control.
*Defined by physical examination.

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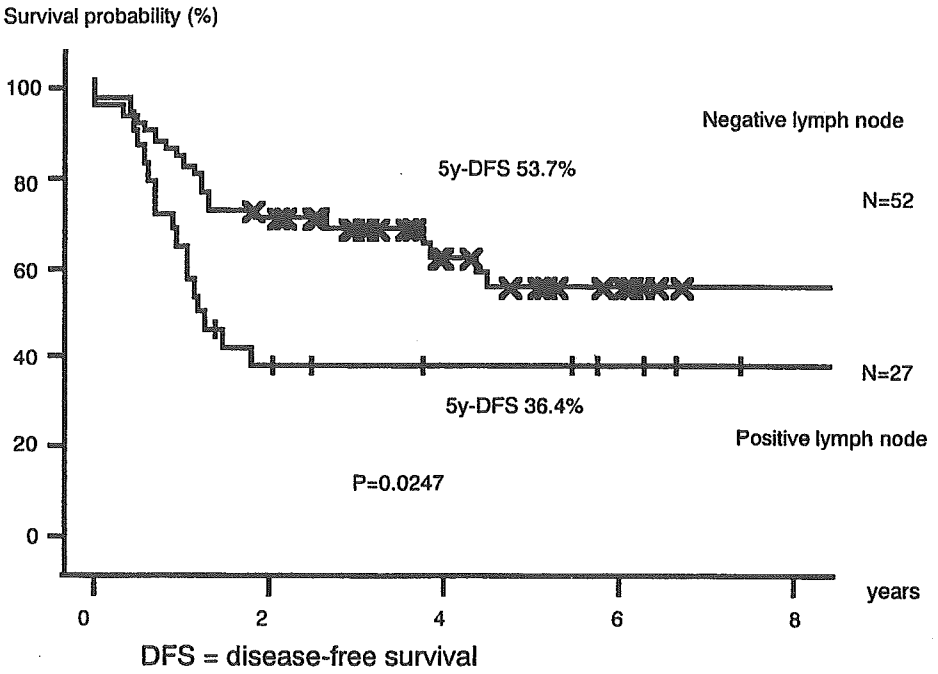

FIGURE 1. Disease-free survival curves of the group divided by lymph nodes.

node showed no apparent correlation to PC. Patients with bilateral disease fixation tended to have a poorer PC compared to those with unilateral disease ($p = 0.11$). Only patient age less than or equal to 50 years proved to be a significantly unfavorable factor for PC ($p < 0.0001$). Elevation of the tumor marker of SCC-antigen had a tendency toward better PC compared to those within the normal range ($p = 0.089$).

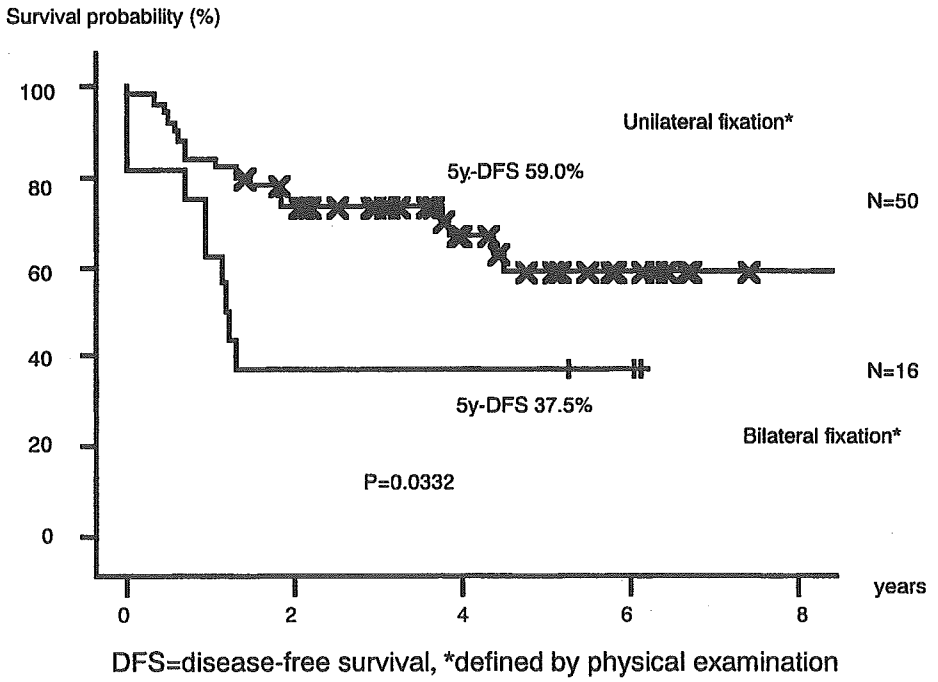

FIGURE 2. Disease-free survival curves of the group divided by pelvic disease fixation (on physical examination).

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Distant Metastasis

Size/volume derived from MRI proved to be a powerful predictor of DMFS. As a function of each parameter of D_{ap}, D_l, D_{cc}, D_{max}, large size proved to be a significantly adverse factor for DMFS (Table 4). For example, patients with disease of D_{max} greater than 60 mm had a significantly poorer DMFS compared to those with D_{max} less than or equal to 60 mm ($p = 0.0307$). Regarding volume analysis, patients with disease volume greater than 100 ml had a significantly worse DMFS compared to those with a smaller volume ($p = 0.0006$, Fig. 3). Positive lymph nodes proved to be a significantly adverse factor for DMFS ($p = 0.0323$). Patients with bilateral pelvic fixation had a significantly poorer DMFS than those with unilateral disease ($p = 0.0168$). Patients aged 50 years or less had significantly lower DMFS than those aged 50 years or more ($p = 0.004$). Prolongation of OTT (>60 days) was revealed as a significantly adverse factor for DMFS ($p = 0.0034$).

Multivariate Analysis

Large calculated volume (>100 ml; $p = 0.009$, relative risk (RR) = 4.478, 95% CI = 1.454–13.797), bilateral pelvic fixation ($p = 0.0087$, RR = 3.461, 95% CI = 1.37–8.745), and patient age (≦50 years; $p < 0.0001$, RR = 38.46, 95% CI = 6.711–200) were revealed to be significant prognostic factors for OAS in the multivariate model (Table 5). Four prognostic factors for DFS, of which the p value remained less than or equal to 0.1 in the univariate analysis, were entered in the multivariate analysis. Again, the same three factors proved to be significantly adverse factors: volume greater than 100 ml; $p = 0.0175$, RR = 3.474, 95% CI = 1.265 to 9.886; bilateral pelvic fixation: $p = 0.0311$, RR = 2.411, 95% CI = 1.106 to 5.481; and patient age 50 years or less; $p < 0.0001$, RR = 21.74, 95% CI = 5.025 to 100.0.

Three prognostic factors for PC were entered in stepwise regression analysis, and only patient age 50 years or less proved to be a significantly adverse prognostic factor ($p = 0.0014$, RR = 14.93, 95% CI = 3.226–23.81).

Five factors for DMFS were selected and entered in the multivariate analysis. As a function of size/volume, volume greater than 100 ml was used because it showed the most appropriate results. Significantly unfavorable factors for DMFS proved to be volume greater than 100 ml ($p = 0.0057$, RR = 4.831, 95% CI = 1.581–14.763), positive lymph nodes ($p = 0.0494$, RR = 2.637, 95% CI = 1.003–6.933), and bilateral disease fixation ($p = 0.0055$, RR = 4.032, 95% CI = 1.505–10.801) in the multivariate analysis.

Relationship Between Pelvic Control and Distant Failures

Sixty-one of 80 patients achieved pelvic control in the present series. We tried to evaluate the development of distant metastasis from the viewpoint of PC. Patients with PC had a significantly better DMFS 5 years: 72.2%, 95% CI = 59.9–84.5%) compared to those without PC (38.0%, 95%

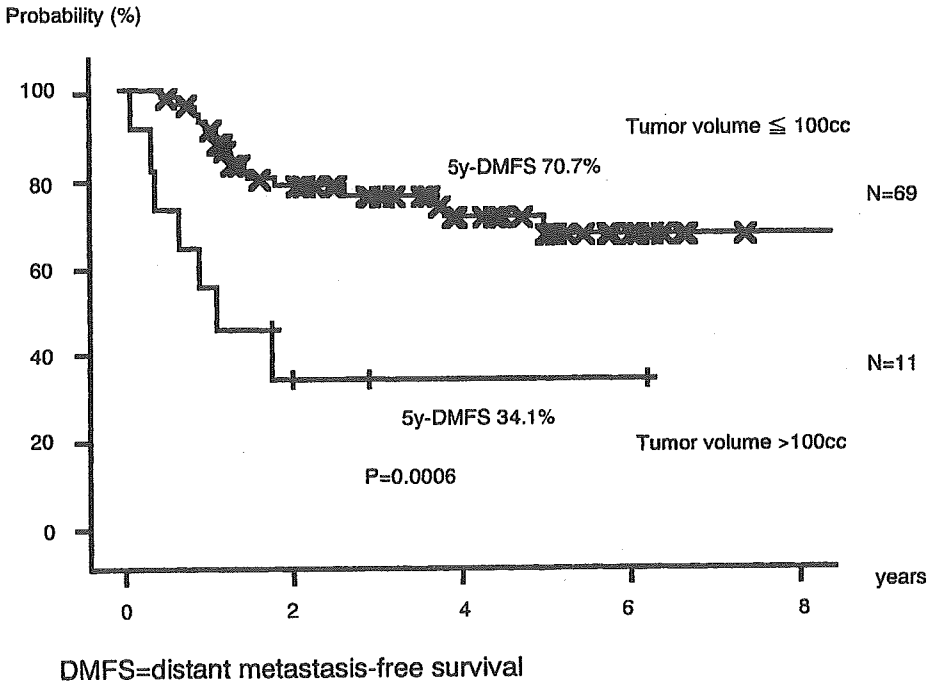

FIGURE 3. Distant metastasis-free survival curves of the group divided by tumor volume.

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TABLE 5. Multivariate analyses of the clinical outcome according to predictive variables (final stepwise-regression model)

	OAS		DFS		PC		DMFS	
Factors	RR	*p* value	RR	*p* value	RR	*p* value	RR	*p* value
Volume (>100 cm^3)	4.478	0.009	3.474	0.0175	NE	NE	4.831	0.0057
Lymph node (positive)	NE	NE	NE	NE	NE	NE	2.637	0.0494
Pelvic fixation (bilateral)*	3.461	0.0087	2.411	0.0311	NE	NE	4.032	0.0055
Patient age (≤50 yr)	38.46	<0.0001	21.74	<0.0001	14.93	0.0014	NE	NE

DFS, disease-free survival; DMFS, distant metastasis-free survival; NE, not entered; OAS, overall survival; PC, pelvic control; RR, relative risk.
*Defined by physical examination.

CI = 10.8–65.2%; p = 0.0003). Even if PC was achieved, 27.8% of these patients experienced distant failures.

In the analysis of patients with PC, tumor size/volume still had a significant influence on DMFS. For example, patients with disease volume greater than 100 ml had a significantly poorer DMFS (41.7%, 95% CI = 7.8–75.6%) compared to those with volume less than or equal to 100 ml (77.6%, 95% CI = 65.1–90.1%; p = 0.0018). Among patients with PC, positive lymph nodes remained a significantly adverse factor for DMFS (55.6%, 95% CI = 32.2–78.9% vs. 78.7%, 95% CI = 64.0–93.4%; p = 0.0124). Bilateral pelvic fixation showed no apparent influence on DMFS in the analysis in same cohort.

Toxicity

Six patients (7.5%) developed late bladder adverse effects 487 to 1,225 days (median 827 days) after the initial treatment. The grades decided by Radiation Therapy Oncology Group (RTOG)/European Organization for Research and Treatment of Cancer (EORTC) score[22] were three with grade I and three with grade II. Late rectal adverse effects developed in 10 patients (12.5%) 211 to 1,092 days (median 391 days) after the initial treatment. The RTOG/EORTC scores of the late rectal adverse effects of grade I, II, III, and IV were four, four, one, and one patient, respectively. Radionecrosis at the pubic bone and femoral head, respectively, developed in two patients.

DISCUSSION

Tumor volume is believe to be an independent prognostic factor of the outcome for patients with cervical carcinoma.[1–3,23–25] Compared to physical pelvic examination, diagnostic imaging such as CT, ultrasonography, and MRI are able to provide accurate tumor size/volume, and these methods are reproducible and independent of interphysician variability.

Among these modalities, MRI also has great benefits in terms of excellent soft tissue contrast resolution, three-dimensional measurement (including volumetric analysis), accurate judgment of invasion surrounding normal tissue, and potential evaluation of tissue characteristics.[7,8,26–28] Surgical specimens proved to correlate well with the findings obtained by MRI in cervical cancer.[2,3,6,26,29,30] In addition, lymph node swelling assessed by MRI was reported to be an adverse prognostic factor.[7,8]

In a previous report, we reported that both size/volume and lymph node swelling derived from pretreatment MRI was a powerful predictor of clinical outcome for patients with stage II disease treated with radiotherapy alone.[10] In this analysis, patients with larger size/volume and/or positive lymph node swelling had a significantly unfavorable outcome as a function of survival and tumor control at both locoregional and distant sites. We could classify the risk model predicting disease-free survival, and thus concluded MRI could provide beneficial information regarding practical outcome among patients with stage II disease. Considering these encouraging data, we again tried to assess the usefulness of MRI among patients with stage III disease. In the present analysis, the MRI protocol and calculated technique was quite similar to that of a previous report.[10]

In contrast to stage II disease, large size/volume showed only a weak impact on OAS/DFS. Large volume remained a significantly unfavorable factor for OAS/DFS in multivariate analysis, although it only showed a tendency as an adverse factor for survival in univariate analysis. Further analysis revealed interesting findings that the failure pattern was markedly different in the analysis of size/volume.

We did not find any correlation between PC and size/volume in the present analysis, but the size/volume significantly influenced the development of distant metastasis. Our cohort included a relatively small patient number. In two institutes, almost half of the consecutive patients received chemoradiotherapy, so they were excluded from this analysis. These biases would have influenced the outcome. However, we suspect that there was a limitation in MRI's detectability for pelvic fixation in stage III disease.

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One explanation is that bilateral fixation showed a tendency as a prognostic factor for PC, but not size/volume. Pelvic fixation was confirmed on physical examination, which is thought *not* to be reproducible and reliable because of interphysician variability. However, *objective* size/volume derived from MRI failed to predict PC in our research on stage III disease. In addition, the size/volume between unilateral fixation and bilateral fixation disease was not significantly different. We therefore expect that MRI is limited in evaluation of disease fixation to the pelvis. This result shows good contrast with that of stage II disease.[10] Indeed, there are many reports from operative cases in which MRI showed excellent ability to represent parametrial invasion.[2,6,26,29,30] However, this is *qualitative*, not *quantitative*. An accurate status of pelvic fixation for stage III disease can hardly be assessed in the same manner, because such locally advanced lesions cannot be resected. Disease with hydronephrosis is believed to be reflected by advanced parametrial involvement. Unfortunately, we could not find any correlation between our results and the presence or absence of hydronephrosis. This is thought to be due to the weak statistical power because of the relatively small number of patients. Advanced disease fixed to the pelvis is not easily controlled by standard radiotherapy,[31–34] because coverage for sidewall disease invasion within a high-dose area via intracavitary brachytherapy becomes more disadvantageous.[34] Thus, the efficacious use of EBRT in the paracentral region becomes more meaningful for stage III disease.[34] Unfortunately, our present trial has basic problems in the uniformity of the radiation technique. We had great variations in the methods of EBRT, prescription of a paracentral boost, timing and dose of ICBT, and type of brachytherapy source. Although the outcome of PC from three institutes did not differ significantly, we suspect that such parameters may influence PC considerably. Usually, the prescribed dose has been decided empirically by the physician's intuition. To clarify the optimal treatment for stage III patients, we need a more efficacious and *quantitative* modality to assess the pelvic fixation.

On the contrary, the size/volume well correlated with the development of distant failure. The same results were reported by other researchers.[23,35] Even if patients could achieve PC, 27.8% of the group experienced distant failure in the present series. In addition, within the group with PC, size/volume remained a statistically adverse factor for DMFS. Thus, we assume that patients with large size/volume will develop greater subclinical distant metastasis. The size/volume calculated from MRI could thus provide promising information for predicting distant failures for stage III patients.

Another problem is the sensitivity of MRI for lymph node involvement. Forty-seven percent to 66% of patients with stage III disease were reported to have lymph node metastasis via surgical staging.[36,37] In the present analysis, 34.2% (27/79) of patients were diagnosed with positive pelvic nodes, a slightly lower incidence than expected. In addition, the relationship between size/volume and frequency of lymph node swelling remained indistinct. It was reported that larger size/volume tumor had a greater incidence of lymphatic involvement.[38] Thus, we suspect that the prevalence of lymph node metastasis might be underestimated in this study. The low sensitivity of MRI might reflect the indistinct relationship between nodal status and PC, compared to that of stage II patients. In Japan, surgical staging is rarely performed on patients with advanced stage disease, so a more accurate imaging technique such as positron emission tomography would be required to acquire more reliable information.[39,40]

Younger age was a significantly adverse factor for OAS, DFS, PC, and DMFS in this study. The same result was reported by several others.[33,41] Younger patients may have more aggressive and extensive disease.

Recently, concurrent chemoradiotherapy has been the standard treatment for stage II-IVA cervical carcinoma,[11–13,16] but patients entered in a randomized controlled study[11–13] consisted of 46.2% to 70% with stage I-II disease. Patients with paraaortic lymph node metastasis were strictly excluded from the analysis. In addition, only 12.5% to 25% of these patients had positive pelvic lymph nodes using surgical staging. A systemic review also reported same staging distribution.[16] Thus, we cannot easily adapt these benefits of concurrent chemoradiotherapy to more advanced disease cases. For patients with stage III disease, a more effective chemotherapy regimen is required, because the reported data on advanced stage disease are not satisfactory.[11–13] These studies achieved an improvement in locoregional control, but failed to reduce distant failures. One explanation is that the prescribed dose of chemotherapy should be reduced for concurrent administration, leading to insufficient control of distant failures. Management of distant metastasis will become a more important issue in refining the outcome of stage III disease.

CONCLUSION

On the analysis using MRI for patients with stage III disease, the size volume proved to be a significant prognostic factor for DMFS, but not for PC. Lymph node status was a predictor of DMFS and DFS in the present analysis. The major cause of treatment failure among stage III disease patients is distant failure; thus, MRI would provide efficacious information for predicting treatment outcome. However, MRI might have some drawbacks in the assessment of disease involvement to the sidewall, because locoregional events were better reflected by pelvic fixation on physical examination than size/volume analysis using MRI.

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Antitumor activity of new combination chemotherapy with irinotecan hydrochloride and nedaplatin against human cervical cancer cell lines

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Abstract. Antitumor activity of combination chemotherapy with irinotecan hydrochloride (CPT-11) and nedaplatin was compared to that with CPT-11 and cisplatin. *In vitro* cytotoxicity of SN-38 (an active metabolite of CPT-11) in combination with nedaplatin or cisplatin was evaluated using three human cervical cancer cell lines (ME-180, CaSki and SiHa). IC_{50} values of nedaplatin against these three human cervical cancer cell lines were about 2-fold as high as those of cisplatin, indicating somewhat weak cytotoxic effects of nedaplatin. Interactions between two drugs in combination were investigated using a simultaneous-exposure schedule and analyzed by the IC_{50}-based isobologram method. Simultaneous exposure to SN-38 with each platinum preparation showed synergistic and additive effects against ME-180 and SiHa. *In vivo* antitumor effects of CPT-11 in the combination with each platinum were studied using SiHa xenografts. While CPT-11, nedaplatin and cisplatin alone hardly showed any antitumor effects even at the maximum tolerated dose (MTD) levels, the combination chemotherapy with CPT-11 and nedaplatin or cisplatin resulted in significant antitumor effects even at three-quarter MTD of CPT-11 combined with two-third MTD of platinum. All treatments were tolerable for mice, indicating that the combinations did not cause significant enhancement in toxicity. In clinical application, nedaplatin causes a lower incidence of nephropathy and does not require the replacement of a large volume of fluid, which is needed for cisplatin administration, facilitating treatment at the outpatient clinic. In addition, the incidences of digestive disorder, peripheral neuropathy and auditory disorder are lower. These findings suggest that the combination chemotherapy with CPT-11 and nedaplatin for squamous cell cancer of uterine cervix is very useful in clinical practice. A dose-finding study should be conducted.

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Key words: irinotecan, CPT-11, nedaplatin, human cervical cancer cell line, antitumor effect

Introduction

Chemotherapy for cervical cancer has markedly improved since the appearance of cisplatin. In clinical practice, combination chemotherapy with several anticancer agents including cisplatin is commonly prescribed (1-13). However, a standard chemotherapy has not yet been established.

Camptothecin sodium (CPT) is a plant alkaloid extracted from the Chinese tree *Camptotheca acuminata* (14). A semisynthetic derivative of this alkaloid, irinotecan hydrochloride (CPT-11), was developed in Japan (15). *In vivo*, CPT-11 is hydrolyzed into an active metabolite, SN-38, by carboxylesterase. The *in vitro* cytotoxicity of this metabolite is 100-fold that of CPT-11, the maternal compound (16,17). In a phase I study involving weekly administration for 4 weeks, dose-limiting toxicities (DLT) were leukopenia and diarrhea (18). In a phase II study investigating patients with cervical cancer, the response rate was 23.6%. The DLT was leukopenia with mild thrombocytopenia. The incidence of grade ≥3 diarrhea was 19% (19).

Nedaplatin (cis-diammineglycolatoplatinum), a new derivative of cisplatin, was also developed in Japan. In a preclinical study, the antitumor activity of this agent was similar or greater than cisplatin. During monthly intravenous administration of nedaplatin, the DLT was bone marrow suppression related to leukopenia and thrombocytopenia (20). In a phase I study, nephrotoxicity, digestive toxicity, neurotoxicity, and acoustic toxicity caused by nedaplatin were markedly less than those induced by cisplatin (21). In a phase II study investigating patients with cervical cancer, the response rate was 34.2% (22).

It has been reported that combination therapy with CPT-11 and cisplatin for cervical cancer achieves a good response rate (23,24). However, for administering cisplatin, this agent should not be indicated for patients with renal dysfunction, and replacement of a large volume of fluid for diuresis is required. Therefore, admission is needed, and this treatment is stressful for patients. Nedaplatin exhibits similar or more potent effects than cisplatin, and its side effects including nephropathy are milder than those of cisplatin. Therefore, the clinical application of combination therapy with CPT-11 and nedaplatin may be useful for improving and maintaining quality of life (QOL). In this study, we examined *in vitro* and *in vivo* combination therapy with CPT-11 and nedaplatin.

Materials and methods

Tumor cell lines. The human cervical tumors, CaSki (epidermoid carcinoma), ME-180 (epidermoid carcinoma, metastasis to omentum) and SiHa (squamous carcinoma), were purchased from American Type Culture Collection (MD, USA), and maintained in RPMI 1640, McCoy's 5a, and α-MEM (Gibco, NY, USA), respectively, and each contained 10% heat-inactivated fetal bovine serum (Lot 11152102, Hyclone, UT, USA) and antibiotics.

Animals. Athymic nude mice (BALB/c-nu/nu), aged 5 or 6 weeks, were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Mice were housed in an exclusive experimental room and were given sterilized food and water *ad libitum*.

Drugs. CPT-11, SN-38 and nedaplatin (254-S) were provided by Yakult Honsha Co., Ltd. (Tokyo, Japan) and Shionogi Co., Ltd. (Osaka, Japan), respectively. Cisplatin was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan).

In vitro cytotoxicity assay. Cellular growth in the presence or absence of the agents was determined as described previously (25). Briefly, rapidly growing cells were harvested, counted, and inoculated at appropriate concentrations (5000 cells/well) into 96-well microplates (Falcon, CA, USA). The following day, drugs were dissolved in DMSO, diluted with culture medium at various concentrations, and then applied to quadruplicate culture wells. After further 72-h incubation at 37°C and 5% CO_2, the amount of viable cells was determined by the MTT assay (26). Inhibitory concentration of 50% (IC_{50}) was calculated from a dose-response curve. The cytotoxicity tests were performed 2-7 times, and the mean and standard deviations of IC_{50} values were calculated.

The effects of SN-38 in combination with nedaplatin or cisplatin were analyzed using the isobologram method of Steel and Peckam (27), and modified by Kano *et al* (28). In brief, based upon the dose-response curves of SN-38 alone and those of nedaplatin or cisplatin alone, three curves (mode I, mode IIa and mode IIb) and the area surrounded by these lines (envelope of additivity) were constructed. IC_{50} values of SN-38 in combination with various concentrations of nedaplatin or cisplatin were plotted as data points. The X-axis and Y-axis values (x, y) were calculated as follows: x = IC_{50} value of SN-38 in combination/IC_{50} value of SN-38 alone; y = concentration of nedaplatin or cisplatin added in combination/IC_{50} value of nedaplatin or cisplatin alone. The criteria of the isobologram are schematically shown in Fig. 1. When the data points fell within the area of left side, inside, right side of the envelope, and outside the square, the interaction between two drugs was considered to be synergistic, additive, sub-additive, antagonistic, respectively. When the data points fall outside the square shown in Fig. 1, the two drugs have antagonistic interaction.

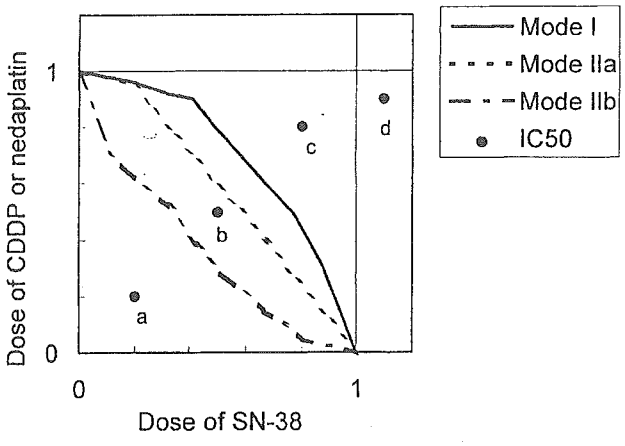

Figure 1. Schematic representation of the isobologram. The envelope of additivity, surrounded by mode I (solid line) and mode II (dotted lines) isobologram lines, was constructed from the dose-response curves of SN-38 and nedaplatin or cisplatin. Data points within the area of left side (a), inside (b), right side (c) of the envelope, and outside the square (d) represent synergistic, additive, sub-additive, and antagonistic interactions, respectively.

Table I. Drug sensitivities of cervical cancer cell lines to each drug.

	IC_{50} ng/ml		
	ME-180	SiHa	CaSki
SN-38	1.34±0.180 (5)	18.2±9.70 (3)	1.76±0.503 (7)
Nedaplatin	409±25.1 (3)	6410±1770 (2)	2790±1060 (4)
Cisplatin	199±23.6 (4)	3010±680 (4)	1280±689 (5)

IC_{50} was determined by MTT assay as described in Materials and methods. The mean and standard deviations were obtained from the data of experiments performed number of times shown in parentheses.

In vivo experiment. SiHa was chosen for *in vivo* study because of its steady growth in nude mice. Tumor masses maintained in nude mice were excised, cut into fragments (ca. mm^3), and transplanted subcutaneously into nude mice (day 0). Estimated tumor volume was calculated according to the following expression: volume (mm^3) = L (mm) x W^2 (mm^2)/2, where L and W represent the length and the width of tumor mass, respectively. When the estimated tumor volume in the

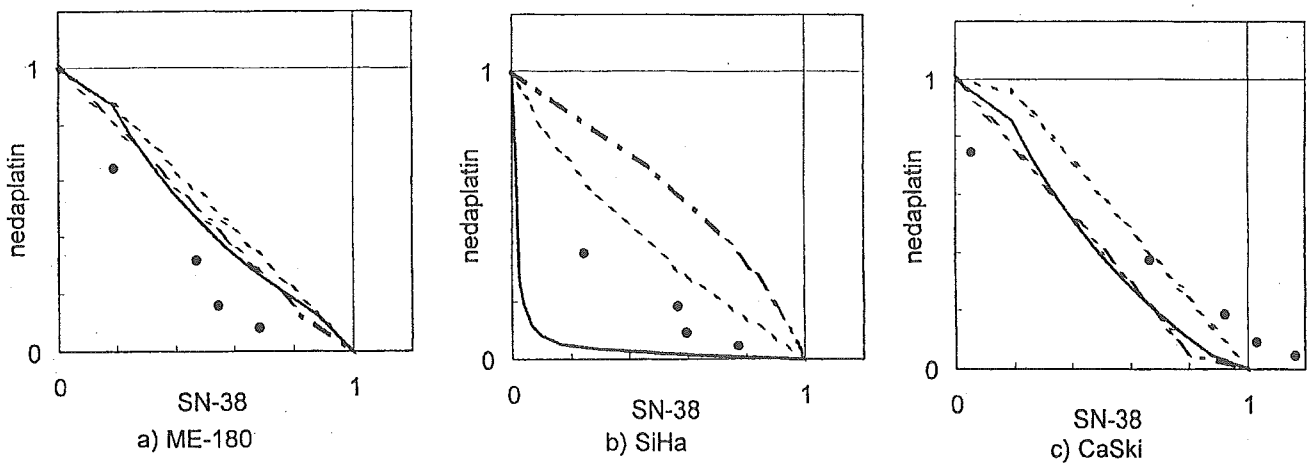

Figure 2. Isobolograms for SN-38 in combination with nedaplatin. The combination of SN-38 and nedaplatin against ME-180 (a) and SiHa (b) resulted in synergistic and additive interactions, respectively. In the case of CaSki (c), the interactions of those two agents varied from synergism to antagonism.

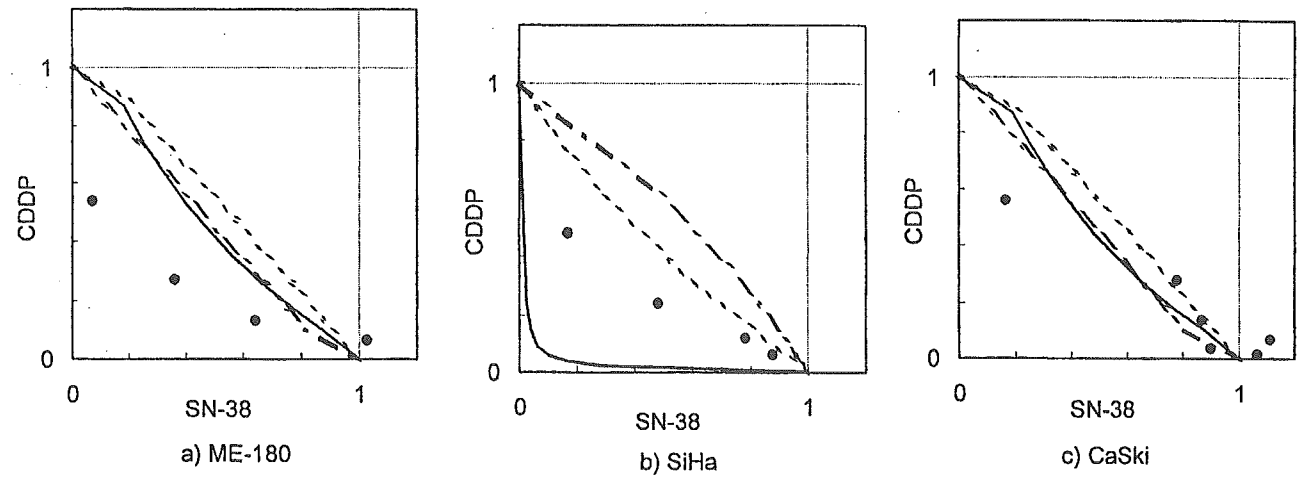

Figure 3. Isobolograms for SN-38 in combination with cisplatin (CDDP). The interaction of SN-38 and cisplatin were synergistic against ME-180 (a) and additive against SiHa (b), while it varied from synergism to antagonism against CaSki (c).

mice had grown to between 100 and 300 mm^3 (day 25), the animals were divided into experimental groups of 6 mice/cage. Mice were treated intravenously with CPT-11 every 4 days for a total of three administrations (q4dx3, on days 25, 29, and 33), and/or with nedaplatin or cisplatin once (qdx1) on day 25. Tumor volume and body weight of the mice were measured two or three times a week for 28 days and then the tumor masses were excised and weighed on day 46. The tumor growth inhibition rate (IR %) was calculated from the following formula: IR (%) = (1 - TWt/TWc) x 100, where TWt represents the mean of tumor weight of treated group and TWc represents that of the control group. When the IR was 58% and higher, the drug was evaluated as effective (29). The rate of body weight loss (BWL) was calculated from the following formula: BWL (%) = (1 - BWn/BWs) x 100, where BWn and BWs represent the mean body weights of mice on day n and on the day of initial administration, respectively. The maximum value of BWL was designated as BWLmax.

Results

Drug sensitivities of cervical cancer cell lines. The sensitivities of three cell lines to SN-38, nedaplatin, and cisplatin, expressed as IC_{50} values, are shown in Table I. ME-180 and CaSki were equally sensitive, but SiHa was less sensitive to SN-38. The mean IC_{50} values of nedaplatin and cisplatin were the lowest to ME-180, and the highest to SiHa. That is, the susceptibility of these cervical cancer cells to platinum was, in decreasing order, ME-180>CaSki>SiHa. The IC_{50} values of nedaplatin against these three cell lines were about 2-fold as high as those of cisplatin, indicating somewhat weak cytotoxic effect of nedaplatin.

Cytotoxicity of SN-38 in combination with nedaplatin or cisplatin. The effects of SN-38 in combination with nedaplatin (Fig. 2) or cisplatin (Fig. 3) varied with the cell lines. The data points in combination of SN-38 with each platinum

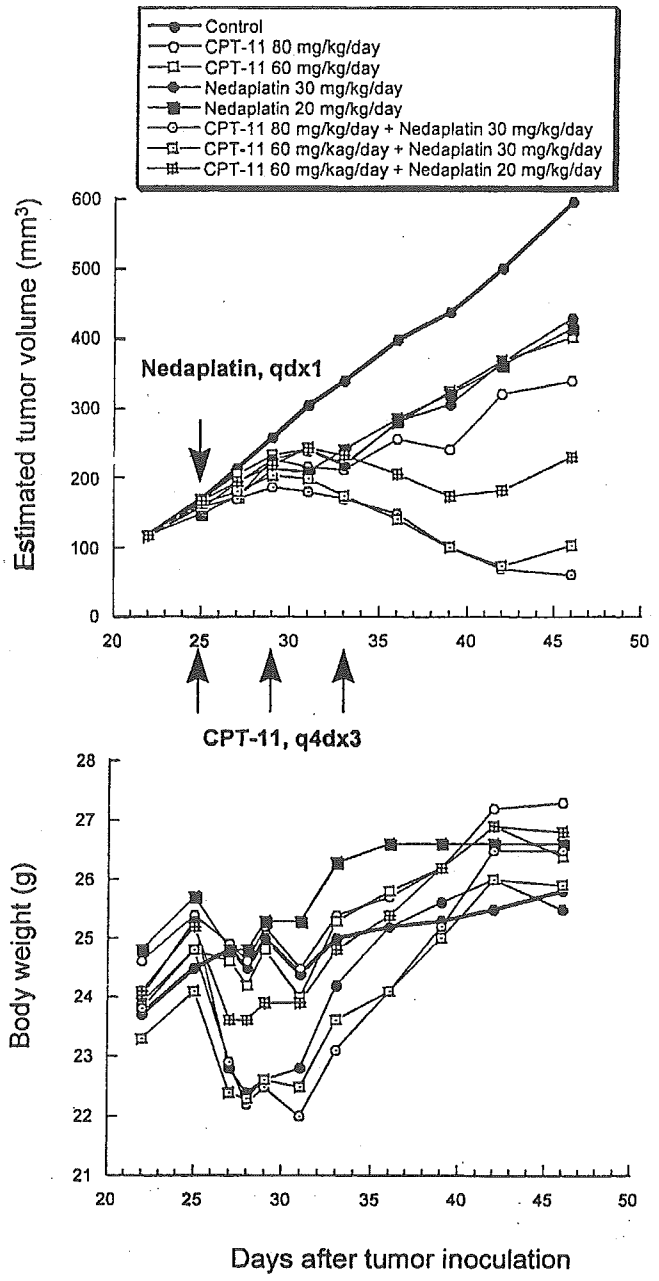

Figure 4. Growth curves of SiHa tumor xenografts and changes in the body weight of mice after treatment with CPT-11 alone, nedaplatin alone, and their combinations. Experimental design is described in Materials and methods. Arrows indicate days of treatment. CPT-11 and nedaplatin alone caused some delay in the growth of SiHa xenografts. Combination therapy with CPT-11 and nedaplatin resulted in significant antitumor effects, which were accompanied by tumor shrinkage at all dose levels examined. The body weight loss in mice given CPT-11 with nedaplatin was similar to that in mice given nedaplatin alone at MTD dose level.

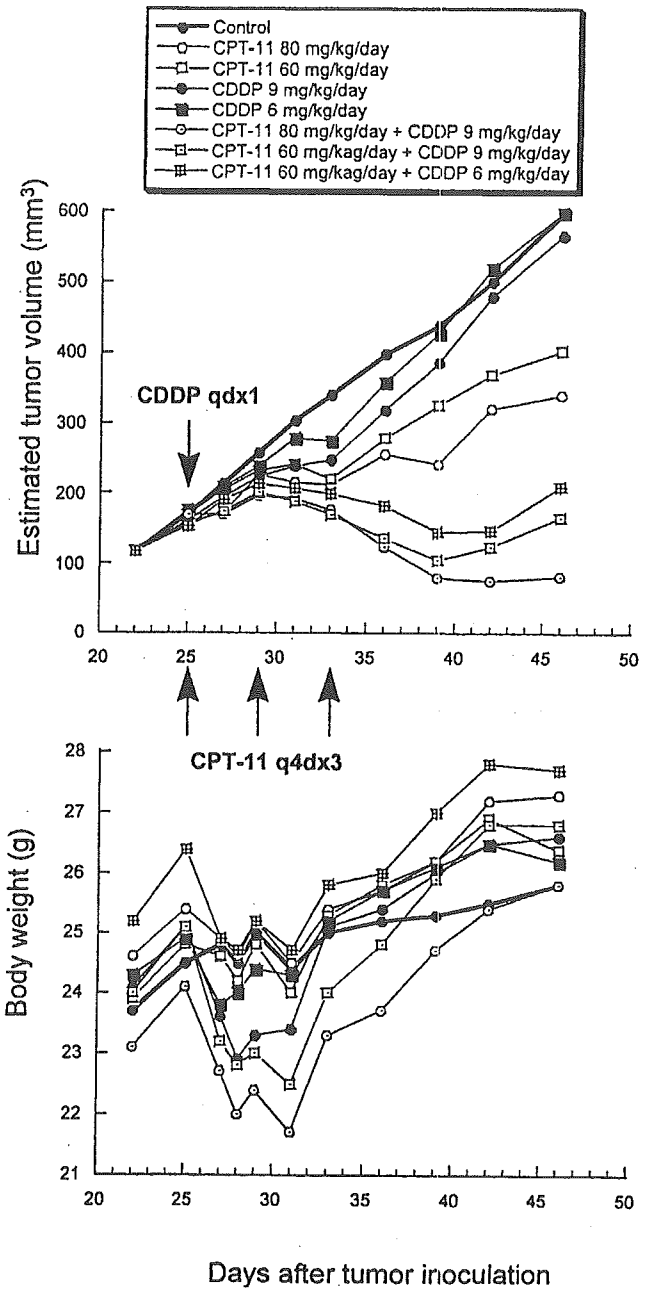

Figure 5. Growth curves of SiHa tumor xenografts and changes in the body weight of mice after treatment with CPT-11 alone, cisplatin (CDDP) alone, and their combinations. Experimental design is described in Materials and methods. Arrows indicate days of treatment. Cisplatin alone failed to show any antitumor effect even at the MTD level (9 mg/kg/day). Combination therapy with CPT-11 and cisplatin resulted in significant antitumor effects accompanied by tumor shrinkage in a dose-dependent manner. The body weight loss in mice given CPT-11 with cisplatin tended to be larger than that in mice treated with the MTD level of cisplatin alone, but it was well tolerated at all dose levels.

preparation against ME-180 fell to the left of the envelope, showing synergistic interaction. Against SiHa, all the data points fell within the envelope, indicating that both combinations resulted in additive interaction. In the case of CaSki, the data points fell into various areas showing synergism to antagonism: antagonistic interactions were observed when SN-38 was combined with a trace of platinum, while synergistic ones were obtained when low dose of SN-38 was combined with comparatively high concentrations of platinum.

Antitumor effect of combination therapy with CPT-11 and nedaplatin or cisplatin against SiHa xenografts in nude mice. Figs. 4 and 5 show the growth curves of SiHa treated with CPT-11 and/or nedaplatin, and with CPT-11 and/or cisplatin, respectively. The results were summarized in Table II. CPT-11, cisplatin, and nedaplatin alone showed no statistically significant antitumor effects. IR % values were 41, 6 and 25% respectively, even at the highest doses, which were selected

Table II. Antitumor effect of CPT-11 in combination with CDDP or nedaplatin against human cervix cancer SiHa in nude mice.

Compound	Total dose mg/kg (Schedule)	Tumor weight		BWLmax % (Day)	No.[a]
		Mean ± SE (g)	IR (%)		
Control	0	0.585±0.100	0	0.3 (31)	0/6
CPT-11	240 (q4x3)	0.349±0.056	41	3.7 (31)	0/6
	180 (q4dx3)	0.433±0.119	26	3.1 (31)	0/6
CDDP	9	0.552±0.073	6	9.0 (28)	0/6
	6	0.648±0.069	-11	4.4 (27)	0/6
Nedaplatin	30	0.437±0.072	25	11.2 (28)	0/6
	20	0.379±0.115	35	3.7 (27)	0/6
CPT-11 + CDDP	240 (q4dx3) + 9 (qdx1)	0.072±0.024[b,c]	88	10.0 (31)	0/6
	180 (q4dx3) + 9 (qdx1)	0.151±0.038[d]	74	10.5 (31)	0/6
	180 (q4dx3) + 6 (qdx1)	0.183±0.054[d]	69	6.4 (28)	0/6
CPT-11 + nedaplatin	240 (q4dx3) + 30 (qdx1)	0.059±0.023[b,e]	90	11.2 (31)	0/6
	180 (q4dx3) + 30 (qdx1)	0.079±0.015[b,e]	87	7.6 (28)	0/6
	180 (q4dx3) + 20 (qdx1)	0.216±0.040[e]	63	6.2 (27)	0/6

Tumor fragments were transplanted into BALB/c-nu/nu mice (day 0). CDDP or nedaplatin was injected i.v. once on day 25, and CPT-11 was injected i.v. three times at 4 day intervals, on day 25, 29, and 33. Tumor weight was assessed on day 46. Tumor weight of all combination therapeutic groups were significantly reduced in comparison with that of control group ($p<0.001$, by Dunnett's test). [a]Number of mice that died of toxicity/number of mice used. [b]$p<0.05$ level of significance vs. CPT-11 180 mg/kg by Tukey-Kramer's test. [d] and [c]$p<0.01$ and 0.001 levels of significance vs. CDDP 9 mg/kg by Tukey-Kramer's test. [e]$p<0.05$ level of significance vs. nedaplatin 30 mg/kg by Tukey-Kramer's test.

as the maximum tolerated dose (MTD) levels in our preliminary dose-finding tests. In contrast, combination therapy with CPT-11 and cisplatin or nedaplatin resulted in significant antitumor effects. IR (%) values in the combination groups were about 90% at the highest dose levels. The antitumor effects were dependent on the doses of two drugs combined, but IR (%) values beyond 58% were obtained even at the lowest dose levels in the combination, three-quarter MTD of CPT-11 combined with two-third MTD of platinum. All treatments were tolerable for mice. The body weight loss in mice given CPT-11 with cisplatin or nedaplatin was similar to that in mice given each platinum preparation alone, indicating that the combinations did not cause significant enhancement in toxicity.

Discussion

To improve antitumor effects, anticancer agents with different mechanisms of action should be combined. The mechanism of action of CPT-11 is speculated to be as follows: CPT-11 or SN-38 forms a complex with DNA topoisomerase I, stabilizes the complex, and inhibits additional binding of DNA, thus inhibiting DNA synthesis (17). Tumoricidal effects are specifically observed during the S-phase and SN-38 demonstrates these effects in a time-dependent manner. Nedaplatin produces its antitumor effects by binding to DNA base after uptake into tumor cells and inhibiting DNA replication. In this respect, this drug has a similar mechanism of action to that of its analogue cisplatin. Further, it has been confirmed that the type of combined bases of nedaplatin after reactions with DNA are identical to those observed in cisplatin.

It is speculated that the synergistic effects of combination therapy with CPT-11 and cisplatin appear because SN-38 significantly inhibits the removal of platinum DNA adducts (30). With regard to the interaction between CPT-11 and nedaplatin, a similar mechanism is assumed.

Isobologram, which was used for *in vitro* evaluation in this study, is advantageous in that it can be managed even when the dose-response curve for anticancer agents does not show first-order kinetics or sigmoid curve (28). Of 3 cell lines of human squamous cervical cancer, antagonistic actions against CaSKi were observed. However, effects on ME-180 and SiHa were synergistic and additive, respectively. Combination therapy with CPT-11 and nedaplatin may be as effective as combination therapy with CPT-11 and cisplatin. In addition, to examine toxicity related to combination therapy, we conducted an *in vivo* experiment using SiHa. This administration method was established based on the administration method for combination therapy with CPT-11 and cisplatin (CPT-11: day 1, 8, 15; cisplatin: day 1) used in clinical practice (23,24). With respect to results, the efficacy of combination therapy with CPT-11 and nedaplatin was similar to that of combination therapy with CPT-11 and cisplatin. The toxicity of combination therapy with CPT-11

and nedaplatin was also similar to that of combination therapy with CPT-11 and cisplatin in this murine model. Kanzawa *et al* also reported the synergistic interaction of CPT-11 and nedaplatin against two lung cancer cell lines (SBC-3 and PC-14) (31).

In clinical application, however, nedaplatin causes a lower incidence of nephropathy, and does not require the replacement of a large volume of fluid, which is needed for cisplatin administration, facilitating treatment at the outpatient clinic. In addition, the incidences of digestive disorder, peripheral neuropathy, and auditory disorder are lower. Therefore, nedaplatin may improve and maintain QOL for patients.

These findings suggest that combination therapy with CPT-11 and nedaplatin for squamous cell cancer of the uterine cervix is very useful in clinical practice. A dose-finding study should be conducted.

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Vaccination with Predesignated or Evidence-Based Peptides for Patients with Recurrent Gynecologic Cancers

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Abstract: Two different trials of peptide vaccination were conducted for patients with recurrent gynecologic cancers. In the first regimen, four HLA-A24$^+$ patients (two with cervical cancer and two with ovarian cancer) were vaccinated with peptides that were predesignated before vaccination. Three patients exhibited with a grade 1 adverse effect, and no clinical response was observed in any patients. In the second regimen, six HLA-A24$^+$ and four HLA-A2$^+$ patients (five with cervical cancer, one with endometrial cancer, one with uterine sarcoma, and three with ovarian cancer) were vaccinated with peptides (maximum four) to which preexisting cytotoxic T lymphocyte precursors in the periphery were confirmed before vaccination. With this regimen, grade 1 adverse effects were observed in eight patients, a grade 2 adverse effect in one patient, and a grade 3 adverse effect (ie, rectal bleeding) in one patient. However, this regimen was able to enhance peptide-specific cytotoxic T lymphocytes in seven of ten patients, and three of five cervical cancer patients showed objective tumor regression. Analysis of immunoglobulin G -reactive to administered peptides suggested that the induction of peptide-specific immunoglobulin G was correlated with clinical responses. Overall, these results suggest that peptide vaccination of patients showing evidence of preexisting peptide-specific cytotoxic T lymphocyte precursors could be superior to vaccination with predesignated peptides, and that the evidence-based regimen is applicable for clinical trials in treatment of patients with recurrent gynecologic cancers.

Key Words: peptide, vaccination, immunotherapy, gynecologic cancer

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Recent advances in molecular biology and tumor immunology have resulted in identification of many tumor antigens and epitopes recognized by tumor-reactive cytotoxic T lymphocytes (CTLs).[1,2] In the field of gynecology, vaccination has been conducted with human papilloma virus (HPV)16 E7-derived peptides for HLA-A2$^+$ patients with cervical cancer, although clinical responses have been unsatisfactory.[3–5] We previously identified a panel of antigenic peptides having the potential to induce peptide-specific and tumor-reactive CTLs in patients with epithelial cancers,[6–16] and several antigens have been shown to be expressed in gynecologic cancers and to have the potential to induce CTLs reactive to gynecologic cancers.[17]

In most protocols of peptide-based vaccination, no consideration has been paid to whether or not peptide-specific CTL precursors are preexistent in cancer patients. Since priming of naive CTLs generally takes longer than boosting of primed CTLs, vaccination with peptides after confirmation of preexisting peptide-specific CTL precursors might be therapeutically beneficial because it would promptly induce peptide-specific CTLs. To put this idea into practice, we developed a new culture protocol to screen a panel of antigenic peptides with a limited number of peripheral blood mononuclear cells (PBMCs),[18] and we confirmed that peptide-specific CTL precursors can be detected in most patients with pancreatic or gastric cancer.[19,20] In this study, gynecologic cancer patients were vaccinated with peptides according to two different regimens: vaccination with predesignated peptides or vaccination with peptides to which preexisting CTL precursors in the periphery were confirmed before vaccination. Our results suggest that the latter evidence-based regimen is effective for patients with recurrent gynecologic cancers, especially cervical cancer.

MATERIALS AND METHODS

Patients and Eligibility Criteria

Two different regimens were approved by the Institutional Ethical Review Boards of Kurume University. Com-

plete written informed consent was obtained from all patients at the time of enrollment. According to the protocol, patients were required to be positive for either HLA-A2 or HLA–A24. The expression of HLA-A24 or HLA-A2 molecules on PBMCs of cancer patients was first determined by flow cytometry, and HLA-A2 subtypes were determined by the sequence-specific oligonucleotide probe method. All patients were pathologically confirmed to have gynecologic cancer (cervical cancer, endometrial cancer, uterine carcinosarcoma, or ovarian cancer). Eligibility criteria included an age of 85 years or younger, serum creatinine of <1.4 mg/dL, bilirubin of <1.5 mg/dL, platelet count of ≥100,000/μL, hemoglobin of ≥8.0 g/dL, total WBC of ≥3000/μL, and negativity for hepatitis B and hepatitis C antigens. All patients had been untreated for at least 4 weeks before the study, and had an Eastern Cooperative Oncology Group performance status of 0 to 1. Patients with evidence of serious illness, an active secondary malignancy during five years before entry, immunosuppression, or autoimmune disease were excluded from the study.

Screening of Peptide-Specific CTL Precursors

Thirty milliliters of peripheral blood was obtained before and after the third, sixth, ninth, and twelfth vaccinations, and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method.[18] Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of a peptide in 200 μL of culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (GIBCO BRL), 10% FCS, 100 U/mL of interleukin-2 (IL-2), and 0.1 μM MEM nonessential amino acid solution (GIBCO-BRL). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) every three days. After incubation for twelve days, these cells were harvested and tested for their ability to produce IFN-γ in response to CIR-A2402 or T2 cells that were preloaded with either a corresponding peptide or HIV peptides (RYLRQQLLGI for HLA-A24 and LLFGYPVYV for HLA-A2) as a negative control. The level of IFN-γ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/mL). All assays were performed in quadruplicate. A two-tailed Student's *t* test was used for the statistical analyses. Based on the results of this test, up to four positive peptides were selected for each patient, and then a skin test was performed. Peptides, which were negative for the skin test, were vaccinated into cancer patients. To evaluate the effects of immunization and newly determined peptides for vaccination, patients were re-screened for peptide-specific CTL precursors after the sixth and the twelfth vaccinations.

Peptides and Vaccination

The peptides used in the present study were prepared by Multiple Peptide Systems (San Diego, CA) under the conditions of Good Manufacturing Practice. The sequences of the peptides are shown in Table 1. All of these peptides have previously been shown to induce HLA-A24- or HLA-A2-restricted and tumor-reactive CTLs in PBMCs of cancer patients.[6–16] Although all peptides for HLA-$A2^+$ patients were selected based on the binding motif to HLA-A*0201 molecules, these peptides are immunogenic not only in HLA-A*0201 patients but also in those with other HLA-A2 subtypes such as HLA-A*0206 or HLA-A*0207.[13–16] Montanide ISA-51 adjuvant was manufactured by Seppic, Inc. (Franklin Lake, NJ). The peptides were supplied in vials containing three mg/mL sterile solution for injection. Three milligrams of peptide with sterile saline was added in a 1:1 volume to the Monotide ISA-51 and then mixed in a Vortex mixer (Fisher Inc., Alameda, CA). The resulting emulsion was injected subcutaneously in the lateral thigh using a glass syringe. Patients were vaccinated initially with three injections every two weeks to determine the toxicity levels. For the patients with no toxicity, the vaccinations were then given every two weeks after obtaining additional informed written consent.

Delayed-type Hypersensitivity (DTH) Skin Test

A skin test was performed using 50 μg of each peptide injected intradermally in a volume of 100 μL using a tuberculin syringe and a 27-gauge needle. Saline was injected as a negative control. Patients were examined 48 hours after the injection and were considered to be positive if they showed an at least 10-mm-diameter induration or erythema.

^{51}Cr-Release Assay and Targets

Cytotoxic activity was measured using a standard 6-hour ^{51}Cr-release assay.[21] In brief, cryopreserved PBMCs were thawed and cultured in the culture medium. On the 14th day of culture, the cells were harvested and used for the assay. Targets used for the ^{51}Cr-release assay were as follows: SKG-I (HLA-$A24^+$ cervical cancer cells), TOC-2 (HLA-$A2^+$ ovarian cancer cells), QG56 (HLA-$A24^-$ lung cancer cells), and HLA-$A2^+$ PHA-blastoid T cells. To minimize nonspecific killing, 20-fold unlabeled K562 cells were added to each well.

Kinetics of Peptide-Specific CTL Precursors

For kinetic analysis of peptide-specific CTL precursors, pre- and post-vaccination PBMCs were incubated at 1×10^5 cells per well in 96-well U-bottom microculture plates in the presence of a peptide. Cells from each well were harvested at the 14th day of culture and tested for their ability to produce IFN-γ by recognition of peptide-pulsed C1R-A24 or T2 cells. The criteria for positive wells are given in the legend for Table 2.

TABLE 1. Pre-vaccination Screening of Peptide-Specific CTL-Precursors

Peptide	Sequence	Reference	Patient						Positive	Vaccinated Case
<HLA-A24>			EBG-001	EBG-002	EBG-003	EBG-004	EBG-006	EBG-007		
SART1 690	EYRGFTQDF	6	● Ar	○ B	○ B				3/6	2/6
SART2 93	DYSARWNEI	7		○ C		○ AC			2/6	2/6
SART2 161	AYDFLYNYL	7	○ A			○ C	○ Ar		3/6	3/6
SART2 899	SYTRLFLIL	7	○ B		○ A			● E	3/6	2/6
SART3 109	VYDYNCHVDL	8	● A		○ A		○ C		3/6	2/6
SART3 315	AYIDFEMKI	8							0/6	0/6
CypB 84	KFHRVIKDF	9				● A			1/6	0/6
CypB 91	DFMIQGGDF	9			○ B	○ E	○ E		3/6	3/6
lck 208	HYTNASDGL	10	○ A	○ A				○ D	3/6	3/6
lck 486	TFDYLRSVL	10		○ A		○ CCC		○ D	3/6	3/6
lck 488	DYLRSVLEDF	10		● Ar					1/6	0/6
ART1 170	EYCLKFTKL	11					● ArA		1/6	0/6
ART4 13	AFLRHAAL	12			● B			● B	2/6	0/6
ART4 75	DYPSLSATDI	12	○ B				○ E		2/6	2/6
<HLA-A2>				EBG-101	EBG-102	EBG-103	EBG-194			
SART3 302	LLQAEAPRL	13			○ AA				1/4	1/4
SART3 309	RLAEYQAYI	13				● CC			1/4	0/4
CypB 172	VLEGMEVV	14		● Ar					1/4	0/4
CypB 129	KLKHYGPGWV	14							0/4	0/4
lck 246	KLVERLGAA	15				○ A			1/4	1/4
lck 422	DVWSFGILL	15			○ A		○ A		2/4	2/4
MAP 294	GLLFLHTRT	16		○ AC			○ AC		2/4	2/4
MAP 432	DLLSHAFFA	16		○ ABC		○ C	○ AAC		3/4	3/4
WHS 103	ASLDSDPWV	16		○ ArA					1/4	1/4
WHS 141	ILGELREKV	16				○ AC			1/4	1/4
UBE 43	RLQEWCSVI	16							0/4	0/4
UBE 85	LIADFLSGL	16							0/4	0/4
UBE 208	ILPRKHHRI	16							0/4	0/4
HNR 140	ALVEFEDVL	16							0/4	0/4
HNR 501	NVLHFFNAPL	16		○ CC	○ A	○ A	○ AA		4/4	4/4
EIF 51	RIIYDRKFL	16								

White circles indicate that the peptide was positive for the CTL-precursor induction assay and was vaccinated. Black circles indicate that the peptide was positive for the CTL-precursor induction assay but was not administered due to immediate-type hypersensitivity by skin test.

The assay was performed in quadruplicate and was evaluated by the criteria shown in Table 2.

The classification is shown as alphabet and each character represents the result of each well. For example, ABC means that three wells were judged as A, B, C and one well was negative quadruplicate wells.

Detection of Peptide-Specific Immunoglobulin G (IgG)

The serum levels of peptide-specific IgG were measured by ELISA as previously reported.[22] Briefly, a peptide (20 μL/well)-immobilized plate was blocked with BlockAce (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween 20-PBS. One hundred microliters per well of serum samples diluted with 0.05% Tween 20-BlockAce were added to the plate. After a 2-hour incubation at 37°C, the plate was washed and further incubated for two hours with a 1:1000-dilluted rabbit anti-human IgG (γ-chain-specific: DAKO, Glostrup, Denmark). The plate was washed again, 100 μL of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish (En Vision, DAKO) was added to each well, and the plate was incubated for 40 minutes. The plate was washed once again, 100 μL/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of one M phosphoric acid. To estimate peptide-specific IgG levels, the optical density (OD) values of each sample were compared with those of serially diluted standard samples, and

TABLE 2. Classification of CTL Response to Peptides

Classification	*P* Value*	IFN-γ Production†
Ar (armed response)	≤0.1	500 ≤ value
A	≤0.05	50 ≤ value
B	≤0.05	25 ≤ value
C	0.05 < *P* ≤ 0.01	50 < value
D	0.05 < *P* ≤ 0.01	25 ≤ value ≤ 50
E	0.1 < *P* ≤ 0.03	100 ≤ value

*The *P* value was determined by Student's *t* test.
†Specific IFN-γ production (pg/mL) was calculated by subtracting the response to HIV-derived irrelevant peptide.

the values were shown as OD units per milliliter. To confirm the specificity of IgG to the peptide, we cultured 100 μL of sample in the peptide-coated wells to absorb peptide-specific IgG, and determined the levels of peptide-specific IgG in the resultant sample.

Evaluation of Response to Treatment

All known sites of disease were evaluated every three months by CT scan or MRI, and/or x-ray examination before and after vaccinations. However, additional examinations were performed when the clinical conditions changed. Patients were assigned a response category according to the response evaluation criteria in solid tumors, based on the June 1999 revision of the WHO criteria published in the WHO Handbook for Reporting Results of Cancer Treatment. Tumor size was evaluated by the longest diameter, and tumor regression of more than 30% for four weeks was regarded as a partial response (PR). The levels of tumor markers, including CA125, CA19-9, carcinoembryonic antigen, and SCC, were measured at the Clinical Examination Laboratory at Kurume University.

RESULTS

Demographics of Patients

Four and 10 patients with gynecologic cancers were enrolled in two different vaccination regimens, respectively. The demographic details of the patients are shown in Table 3. The median age of these patients was 53.5 years (range, 38–68 years). Four HLA-A24$^+$ patients (2 with cervical cancer and 2 with ovarian cancer) were enrolled in the first vaccination regimen, in which predesignated peptides were vaccinated based on the finding that SART2 and ART4 antigens were identified using HLA-A24-restricted CTLs reactive to squamous cell carcinoma and adenocarcinoma, respectively.[7,12] The other 6 HLA-A24$^+$ and 4 HLA-A2$^+$ patients (five with cervical cancer, three with ovarian cancer, one with endometrial cancer,

TABLE 3. Patient Characteristics

Regime	Case No.	HLA	Age (yr)	PS*	Tumor	Stage	Site of Metastasis	Previous Treatment†	No. of Vaccination Received	Clinical Response
1	SART2-001	A24	67	0	Cervical cancer	IVa	Pelvic LN	C/R, C	9	PD (3M)
1	SART2-002	A24	52	1	Cervical cancer	IIb	Lung, para-aorta LN, pelvic LN, virchow LN	S, C, R	9	PD (5M)
1	ART4-001	A24	52	0	Ovarian cancer	IIc	Multiple liver, multiple LN	S, C	7	PD (1M)
1	ART4-002	A24	68	0	Ovarian cancer	IIIa	Lung, ischial bone	S, C, R	15	PD (3M)
2	EBG-001	A24	40	0	Cervical cancer	Ib	Para-aorta LN	C/R	31	SD (3M), PR (4M), PD (15M)
2	EBG-002	A24	67	0	Endometrial cancer	Ic	Lung	S, C	18	PD (2M)
2	EBG-003	A24	66	0	Cervical cancer	IIb	(−)	S, C	25	SD (3M), PD (6M)
2	EBG-004	A24	57	0	Ovarian cancer	IIIc	(−)	S, C	13	SD (8M)
2	EBG-006	A24	56	0	Uterine carcinosarcoma	III	(−)	S	8	SD (3M), PD (5M)
2	EBG-007	A24	38	0	Cervical cancer	IIIb	Lung	C/R, C	7	PD (2M)
2	EBG-101	A2 (A*0206)	67	0	Cervical cancer	IVa	(−)	C, R	10	SD (3M), PR (10M)
2	EBG-102	A2 (A*0206)	49	0	Ovarian cancer	IIIc	(−)	S	8	PD (3M)
2	EBG-103	A2 (A*0201)	63	0	Cervical cancer	IVa	Parametrium	R	14	SD (13M)
2	EBG-104	A2 (A*0201)	59	1	Ovarian cancer	IIIc	Spleen	S, C	5	PD (2M)

*Performance Status by ECOG score.
†S, surgery; C, chemotherapy; R, radiation therapy; C/R, chemoradiotherapy; LN, lymph node.

and one with uterine sarcoma) were enrolled in the second regimen, in which patients were vaccinated with peptides to which preexisting CTL precursors were confirmed before the peptide vaccination. Three patients (EBG-003, EBG-004, and EBG-006) had no measurable disease at the time of entry but were enrolled in this study because they had high risk of relapse (EBG-003, cervical cancer stage IIb post chemotherapy-radiotherapy; EBG-004, ovarian cancer stage IIIc recurrence postchemotherapy; and EBG-006, uterine carcinosarcoma stage III post simple total abdominal hysterectomy and bilateral salpingo-oophorectomy). No patient had received any concurrent treatments, or any steroids, or any other immunosuppressive drugs, for 4 weeks prior to the vaccination. All 10 patients completed the first three vaccinations within the protocol under informed consent, and all of them received additional vaccinations (5 to 31) after providing additional informed consent.

Toxicities

All patients were evaluated for toxicity levels. The overall toxicities are shown in Table 4. In the first regimen, local redness and swelling were observed in 3 of 4 patients, and no other toxicity was observed. In the second regimen, common adverse events were local redness and swelling (grade 1 or 2) at the injection sites. Fever was observed in 3 patients. Inguinal lymph node swelling (grade 1) was observed in one patient. Although rectal bleeding (grade 3) was observed in one patient (EBG-101) after the fifth vaccination, the correlation to the vaccination was unclear because this patient had radiation-induced colitis in the rectum before entry into this trial. In addition, there was no clinical evidence of autoimmune reactions as determined by symptoms, physical examination, or laboratory tests.

First Regimen

In the first regimen, patients were vaccinated with predesignated peptides. Although we had designated 3 SART2 peptides (SART2 93, SART2 161, and SART2 899) as peptides for two patients with cervical cancer (SART2-001 and SART2-002), the SART2 899 peptide was not vaccinated because this peptide elicited immediate-type hypersensitivity at the skin test (data not shown). The other two patients with ovarian cancer (ART4-001 and ART4-002) were vaccinated with only the ART4 75 peptide because the ART4 13 peptide also elicited immediate-type hypersensitivity at the skin test (data not shown).

Screening of Peptide-Specific CTL Precursors and the Second Regimen

In the second regimen, prevaccination PBMCs were used for screening of preexisting CTL precursors reactive to peptides. Fourteen peptides binding to HLA-A24 molecules and 16 peptides binding to HLA-A2 molecules were used for the screening. The results for each well were classified into 6 groups based on the P value and the amounts of IFN-γ, as shown in Table 2. Up to 4 peptides were selected as candidates for the peptide vaccination. Patients who showed immediate-type hypersensitivity by the skin test were vaccinated with the fifth-ranked peptide, provided that their skin test result for this peptide was negative. The results of prevaccination screening of peptide-specific CTL precursors are shown in Table 1. In HLA-A24$^+$ patients, the SART2 161, the CypB 91, the lck 208, and the lck 486 peptides were most frequently positive (3 of 6 patients) for CTL precursors without immediate-type hypersensitivity. In HLA-A2$^+$ patients, the HNR 501 peptide was positive in all patients, and the MAP 432 peptide showed the second highest rate of positivity (3 of 4 patients). It is of note that 8 patients were positive for at least 4 peptides, with the exception that EBG-007 and EBG-102 were positive for 2 and 3 peptides, respectively. After the 6th vaccination, peptide-specific CTL precursors were rescreened, and peptide candidates for additional vaccination were determined. In some cases, peptide-specific CTL precursors were screened a third time after the twelfth vaccination, and further peptide candidates for vaccination were determined. All data are summarized in Table 5. The peptide vaccination based on the second regimen augmented peptide-specific IFN-γ production in 7 patients. Unexpectedly, peptide vaccination seemed to induce CTLs reactive to irrelevant peptides. As typically observed in patient EBG-103, the first vaccination with the lck 246, the

TABLE 4. Toxicity Associated With the Peptide Vaccination

Toxicity	Regimen 1					Regimen 2				
	Grade 1	Grade 2	Grade 3	Grade 4	Total	Grade 1	Grade 2	Grade 3	Grade 4	Total
Dermatologic	3				3/4	4	1			5/10
Fever					0/4	3				3/10
Rectal bleeding					0/4			1		1/10
Inguinal lymph node swelling					0/4	1				1/10

Toxicities are based on NIH Common Toxicity Criteria.

TABLE 5. Summary of Responses of the Regimen 2

Case	Peptide	Peptide-specific CTL*			Ab to Peptides		DTH		Clinical Response/Time to Progression (Months)
		Pre	Post (6th)	Post (12th)	Pre	Post†	Pre	Post	
<HLA-A24>									
EBG-001	SART2 161	A‡	–	–	–	–	–	–	shrinkage of metastatic LN (42%)
	SART2 899	B	–	A	+	+ (6)	–	–	CEA; 207 → 105
	lck 208	A	Ar	AC	–	+ (9)	–	–	SD (3M), PR (4M), PD (15M)
	ART4 75	B	C	CCC	–	+ (21)	–	–	
	SARTI 690	Ar	C	ArAr	–	+ (12)	–	–	
	SART3 109	AA	Ar	–	–	+ (5)	–	–	
	lck 488	–	A	–	–	+ (5)	–	–	
	CypB 91	–	–	A	–	–	–	–	
EBG-002	SART1 690	B	–	–	–	–	–	–	PD/2M
	SART2 93	C	–	AA	–	–	–	–	
	lck 208	A	B	–	–	+ (9)	–	–	
	lck 486	A	–	A	–	+ (6)			
	ART1 170	–	–	–	–	–	–	–	
	lck 488	Ar	–	AAAE	–	–	–	–	
EBG-003	SART1 690	B	A	A	–	–	–	–	SD (3M), PD (6M)
	SART2 899	A	–	–	–	–	–	+ (3)	
	SART3 109	A	–	–	–	–	–	–	
	CypB 91	B	–	ArA	–	–	–	–	
	lck 488	–	AAA	–	–	–	–	+ (9)	
	SART2 93	–	BB	–	–	–	–	–	
	SART2 161	–	A	–	–	–	–	–	
	SART3 315	–	–	Ar	–	+ (10)	–	+ (14)	
EBG-004	SART2 93	AC	AC	A	–	–	–	–	SD (8M)
	SART2 161	C	AAA	AB	–	–	–	+ (3)	
	CypB 91	E	–	–	–	–	–	+ (3)	
	lck 486	CCC	–	AA	–	+ (6)	–	–	
	SART3 315	–	A	AAA	–	–	–	+ (7)	
EBG-006	SART2 161	Ar	ArAr	NT	–	–	–	–	SD (3M), PD (5M)
	SART3 109	C	ArArA	NT	–	+ (6)	–	–	
	CypB 91	E	–	NT	–	–	–	–	
	ART4 75	E	–	NT	–	–	–	–	
	SART2 93	–	ArAr	NT	–	–	–	–	
	lck 488	–	C	NT	–	–	–	–	
EBG-007	lck 208	D	–	NT	–	+ (6)	–	–	PD (2M)
	lck 486	D	–	NT	–	–	–	–	
	SART3 109	–	B	NT	–	+ (11)	–	–	
<HLA-A2>									
EBG-101	MAP 294	AC	–	NT	–	–	–	–	Shrinkage of tumor (48%)
	MAP 432	ABC	ArA	NT	–	+ (6)	–	–	SD (3M), PR (10M)
	WHS 103	ArA	A	NT	–	–	–	–	SCC 289 → 36
	HNR 501	CC	–	NT	–	–	–	–	CEA: 13.3 → 6.6
	lck 246	–	ArAr	NT	–	+ (4)	–	–	
	lck 422	–	ArArAr	NT	–	–	–	–	
	UBE 43	–	–	NT	–	+ (4)	–	–	
EBG-102	SART3 302	AA	A	NT	–	–	–	+ (2)	PD (3M)
	lck 422	A	NT	NT	–	–	–	+ (3)	CA 125: 24000 → 17000
	HNR 501	A	–	NT	–	–	–	–	CA 19-9: 113 → 29.3
	SART3 309	–	AA	NT	–	–	–	–	
	MAP 294	–	A	NT	–	–	–	–	
	MAP 432	–	B	NT	–	–	–	–	
EBG-103	lck 246	A	ArC	ArArA	–	+ (5)	–	–	SD (13M)
	MAP 432	C	–	AACC	–	+ (7)	–	–	
	WHS 141	AC	–	–	–	+ (4)	–	–	
	HNR 501	A	B	A	–	–	–	–	
	lck 422	–	ABCC	AAAA	–	–	–	–	
	UBE 43	–	AAAC	ABC	–	+ (2)	–	–	
EBG-104	lck 422	A	NT	NT	–	–	–	–	PD (2M)
	MAP 294	AC	NT	NT	–	+ (3)	–	–	
	WHS 432	AAc	NT	NT	–	–	–	–	
	HNR 501	AA	NT	NT	–	–	–	–	

NT: not tested.
*The criteria are shown in Table 2.
†The number in the parenthesis represents the vaccination when IgG to the peptide was detected for the first time.
‡Peptides shown as a bold letter were administered into patients.

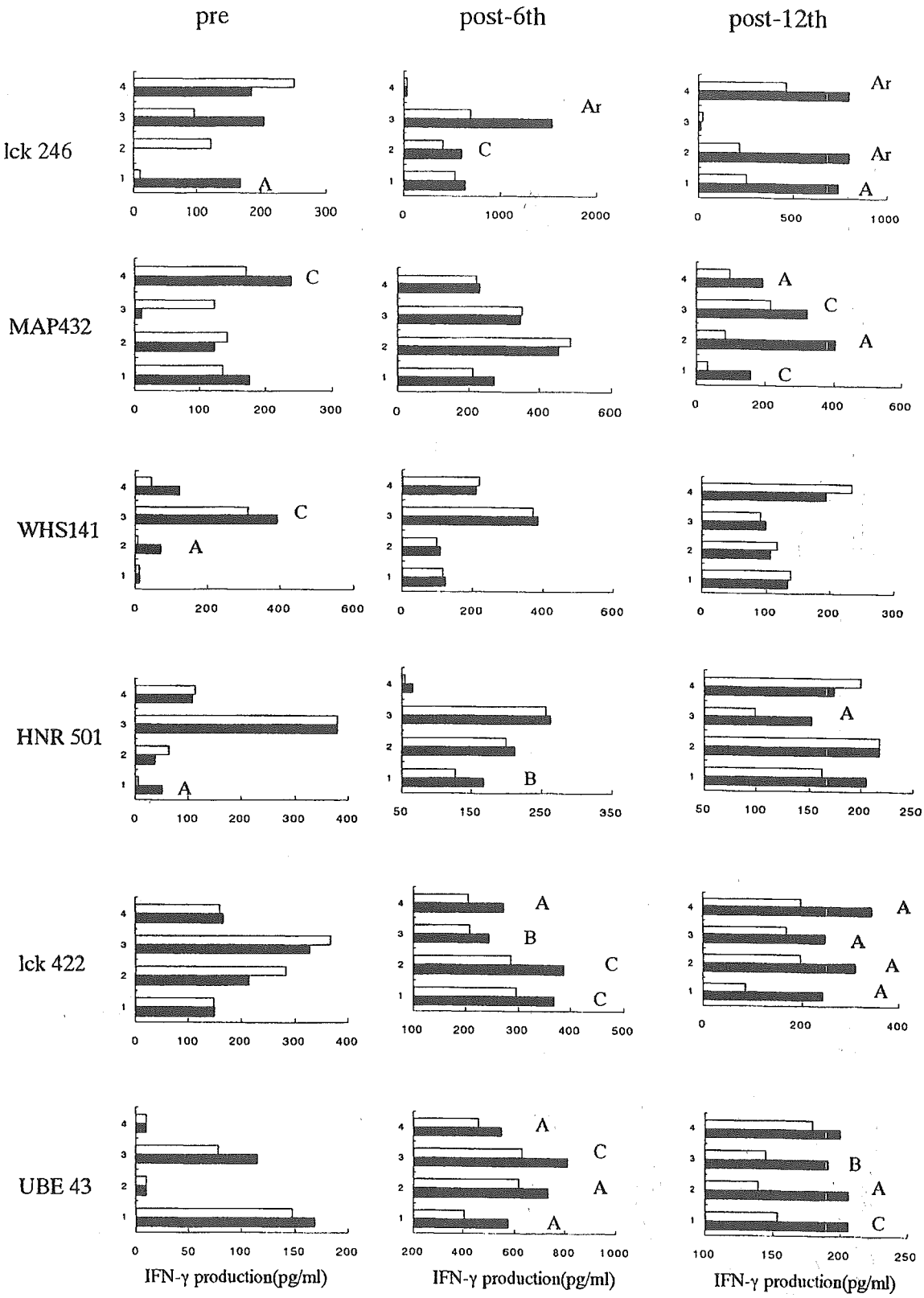

FIGURE 1. Detection of peptide-specific CTL precursors. Pre- and post- (6^{th} and 12^{th}) vaccination PBMCs from patient EBG-103 (HLA-$A2^{+}$) were applied for the screening of peptide-specific CTL precursors. Values represent IFN-γ production by the in vitro cultured PBMCs. Criteria for evaluation are shown in Table 2. Open and closed bars represent IFN-γ production in response to HIV peptide-pulsed and the corresponding peptide-pulsed stimulator cells, respectively. T2 cells were used as stimulator cells.

MAP 432, the WHS 141, and the HNR 501 peptides enhanced CTLs reactive to the lck 246 peptide but also resulted in increased responses of CTLs reactive to both the lck 422 and the UBE 43 peptide, neither of which was administered into the patient (Fig. 1). After the third screening, this patient was vaccinated with the lck 246, the HNR 501, the lck 422, and the UBE 43 peptides, but this vaccination augmented CTLs reactive to the MAP 432 peptide. The same effect was found in the other 7 patients. However, no augmentation of peptide-specific CTLs was observed in patient EBG-007. As for patient EBG-104, the assay of CTL precursors before the sixth vaccination was not performed because the patient died after the fifth vaccination due to progression of disease.

Kinetic Assay of Cytotoxicity of the Second Regimen

Because of the limited availability of samples, we kinetically evaluated anti-tumor cytolytic activity of pre- and post-vaccination PBMCs in only four patients (Fig. 2). In patient EBG-001, CTL activity against SKG-I (HLA-A24$^+$) was augmented transiently after the vaccination, compared with that against QG56 (HLA-A24$^-$) but was subsequently diminished. In patient EBG-007, CTL activity against SKG-I increased after the sixth vaccination. In patient EBG-101, CTL activity against TOC-2 (HLA-A2$^+$) was transiently augmented compared with that against HLA-A2$^+$ PHA blasts. In patient EBG-102, CTL activity against TOC-2 was higher than that against HLA-A2- QG-56 throughout the peptide vaccination.

Serum IgG Specific to Peptides Administered

We also examined whether peptide-specific IgG could be detected in vaccinated patients (Table 5). Peptide-specific IgG was detected before vaccination in only patient, EBG-001. Although no peptide-specific IgG was detected in the other 9 patients before the peptide vaccination, peptide vaccination resulted in the induction of peptide-specific IgG in 9 of 10 pa-

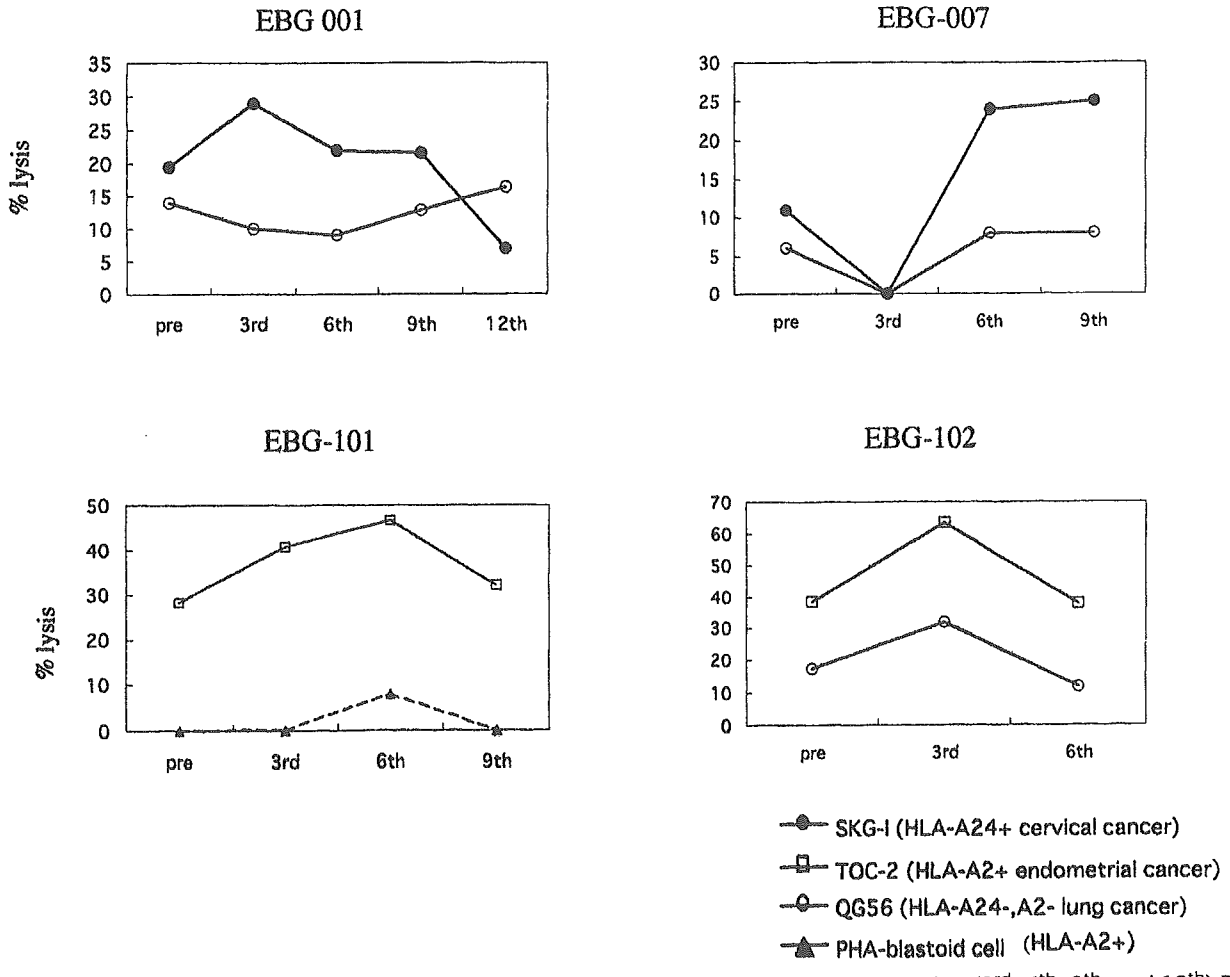

FIGURE 2. Kinetic analysis of cytotoxicity of vaccinated patients. Frozen pre- and post-vaccination (3rd, 6th, 9th, and 12th) PBMCs were thawed and incubated for 14 days with IL-2 alone without any peptides. A cytolytic assay against targets was carried out by a 6-hour ^{51}Cr-release assay at an E/T ratio of 40:1. Values are the means of triplicate assays. Patients EBG-001 and EBG-007 were positive for HLA-A24, and patients EBG-101 and EBG-102 were positive for HLA-A2.