Table 2 Response

	CR	PR	SD	PD	Overall response
Total	5	13	5	0	18 (78.3 %)
TC	1	3	0	0	4 (100 %)
DC	4	10	5	0	14 (73.7 %)

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *TC* paclitaxel + carboplatin, *DC* docetaxel + carboplatin

Table 3 Adverse events of TC/DC therapy

$n = 23$	Grade				
	1	2	3	4	≧3 (%)
Leukopenia	2	9	11	1	12 (52.2)
Neutropenia	1	1	7	14	21 (91.3)
Thrombocytopenia	11	0	0	0	0
Anemia	11	12	0	0	0
Nausea	11	3	1	0	1 (4.3)
Vomiting	5	3	0	0	0
Diarrhea	2	0	0	0	0
Neurotoxicity	18	0	0	0	0
Dyspnea	3	0	0	0	0
Fibrile neutropenia	0	0	2	0	2 (8.7)

TC paclitaxel + carboplatin, *DC* docetaxel + carboplatin

Surgery completion and adjuvant therapy

Radical hysterectomy after NAC was completed in 18 of the 23 patients, giving a surgery completion rate of 78.3 %. Adjuvant therapy after radical hysterectomy consisted of no treatment in 3 cases (13.0 %), radiotherapy in 2 cases (8.7 %), chemotherapy in 15 cases (65.2 %), and CCRT in 3 cases (13.0 %).

Survival

The median follow-up period was 31 months (range 9–90 months). The median progression-free survival period was 26 months (95 % Cl, 13.5–38.5 months), and the median overall survival period was 35 months (95 % Cl, 20.9–49.1 months) (Fig. 1). The 5 patients in whom surgery was not complete died of their primary disease within 35 months. Their median PFS and OS were 8 months (3–12 months) and 21 months (10–35 months), respectively.

Discussion

The incidence of non-squamous cell carcinoma of the uterine cervix has been steadily rising in Japan, currently accounting for approximately 10–15 % of all cervical cancer cases. Lymph node metastasis is more frequent with this disease, compared with invasive squamous cell carcinoma [11], and its sensitivity to radiotherapy and chemotherapy is considered to be lower [12]. Thus, squamous and non-squamous cell carcinomas must be analyzed separately. It is advisable and desirable to try new therapeutic strategies in non-squamous cell carcinoma, but the number of published studies involving this type of cervical cancer is small, with the number of cases analyzed in these reports also small. Thus, no high-level evidence regarding treatment has been obtained for this type of cervical carcinoma.

The response rates of adenocarcinoma are reportedly 20 % to cisplatin [13], 15 % to ifosfamide [14], 14 % to 5-fluorouracil [15], and 12 % to oral etoposide [16]; these response rates are lower than those of squamous cell carcinoma. According to Curtin et al. [17], however, the response rate of adenocarcinoma to paclitaxel is as high as 31 %, even when the agent is used independently. Docetaxel has also been attracting interest as an agent of NAC. Nagao et al. evaluated the efficacy of combined chemotherapy using a DC regimen (docetaxel 60 mg/m^2 and carboplatin at AUC 6 on day 1, repeating the combination every 21 days) in 17 patients with advanced or recurrent cervical cancer, including 6 with adenocarcinoma and 1 with adenosquamous carcinoma. A partial response was obtained in 6 of the 7 cases with adenocarcinoma (including the case of adenosquamous carcinoma); the response rate was 86 % [18]. Considering these findings, we conducted a pilot study involving standard regimens of TC and DC, conventionally used for the treatment of ovarian cancer.

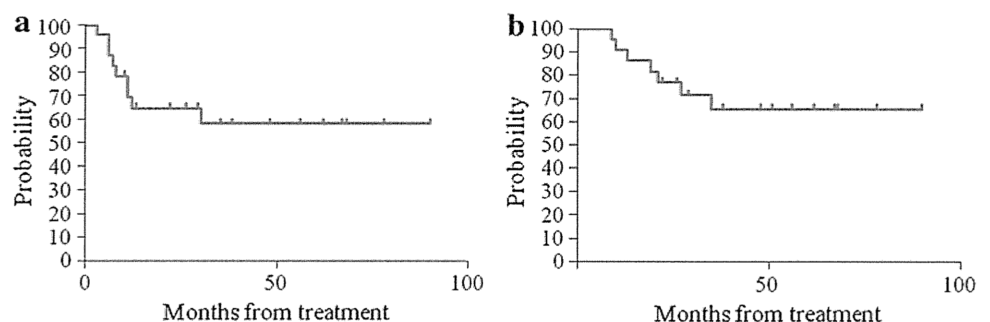

Fig. 1 Kaplan–Meier curves for progression-free survival (**a**) and overall survival (**b**). The median PFS for all patients was 26 months (95 % CI, 13.5–38.5 months), and the median OS was 35 months (95 % CI, 20.9–49.1 months)

Table 4 Treatment results and outcomes of all patients

Patients	Age	Stage	Cell type	Regimen	Cycles	Responses	Surgery	Adjuvant	Follow-up period (months)	PFS (months)	OS (months)	Outcome
1	52	IIb	ASC	DC	2	CR	Incomplete	CCRT	21	12	21	DOD
2	50	Ib2	MAC	TC	2	CR	Complete	NT	90	90	90	NED
3	55	IIb	ASC	DC	2	CR	Complete	CT	62	62	62	NED
4	39	Ib2	MAC	DC	3	CR	Complete	CT	51	11	51	AWD
5	36	Ib2	MAC	DC	2	CR	Complete	CT	22	22	22	NED
6	32	IIb	ASC	DC	3	PR	Incomplete	NT	19	11	19	DOD
7	49	Ib2	MAC	DC	2	PR	Complete	CCRT	78	78	78	NED
8	60	IIb	ASC	DC	2	PR	Complete	NT	68	30	68	AWD
9	54	IIb	EDC	TC	1	PR	Complete	CT	68	68	68	NED
10	40	Ib2	MAC	TC	2	PR	Complete	CT	67	67	67	NED
11	38	IIb	MAC	DC	2	PR	Complete	CT	9	6	9	DOD
12	63	IIb	CCC	DC	2	PR	Complete	RT	48	48	48	NED
13	50	IIb	EDC	DC	2	PR	Complete	CT	35	35	35	NED
14	53	Ib2	EDC	DC	2	PR	Complete	CT	38	38	38	NED
15	54	IIb	MAC	DC	2	PR	Complete	CT	29	29	29	NED
16	52	IIb	MAC	TC	3	PR	Incomplete	CT	27	7	27	DOD
17	45	IIb	EDC	DC	2	PR	Complete	CT	10	10	10	NED
18	51	IIb	EDC	DC	3	PR	Complete	CT	13	13	13	NED
19	45	IIb	MAC	DC	2	SD	Incomplete	CT	10	3	10	DOD
20	52	IIb	ASC	DC	2	SD	Complete	CT	13	6	13	DOD
21	56	IIb	ASC	DC	2	SD	Complete	CCRT	56	56	56	NED
22	61	IIb	ASC	DC	3	SD	Incomplete	RT	35	8	35	DOD
23	45	IIb	MAC	DC	2	SD	Complete	CT	26	26	26	NED

ASC adenosquamous cell carcinoma, *MAC* mucinous adenocarcinoma, *EDC* endometrioid adenocarcinoma, *CCC* clear cell adenocarcinoma, DC docetaxel + carboplatin, *TC* paclitaxel + carboplatin, *CR* complete response, *PR* partial response, *SD* stable disease, *NT* no treatment, *CT* chemotherapy, *RT* radiotherapy, *CCRT* concurrent chemoradiation therapy, *PFS* progression-free survival, *OS* overall survival, *NED* no evidence of disease, *AWD* alive with disease, *DOD* died of disease

In the analysis of adverse events, severe neutropenia developed in 91.3 % of patients, but subsided in response to short-term treatment with a G-CSF preparation. During the first course of DC therapy, grade 3 febrile neutropenia developed in 2 cases; the dose of both agents was reduced for the next course of treatment. All signs, specific to taxanes, of peripheral neuropathy were grade 1 or less, allowing for continuation of treatment while preserving the quality of life of the individual patients. No serious adverse events occurred, and the response rate was 78.3 %. This study demonstrated a high response rate of bulky non-squamous cell carcinoma of the cervix to NAC using taxanes (paclitaxel or docetaxel) and carboplatin. It also demonstrated the safety of the medications in this regimen. The completion rate of radical hysterectomy, however, was only 78.3 %; thus, the treatment outcomes in this study were not satisfactory. Possible reasons for the low surgery completion rate include the rapid progression of non-squamous cell carcinoma, frequent invasion of tissues and organs surrounding the uterus, and frequent lymph node metastasis.

The treatment results and outcomes of all patients were shown in Table 4. Unfortunately, all patients with incomplete surgery ultimately experienced disease recurrence and died of their primary disease. Thus, the significance of NAC at present may not be to prolong survival time. Instead, in our view, NAC should be performed to fully optimize patients' conditions with its antitumor effect in order to improve the chances of complete surgery. Further study is needed regarding the long-term outcomes of NAC.

Conflict of interest The authors have no conflict of interest to declare.

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Obstetrics and Gynaecology Research

The official Journal of Asia and Oceania Federation of Obstetrics and Gynaecology
Japan Society of Obstetrics and Gynecology

doi:10.1111/jog.12068 *J. Obstet. Gynaecol. Res. Vol. 39, No. 8: 1347–1353, August 2013*

Prospective evaluation of the Amplicor HPV test for predicting progression of cervical intraepithelial neoplasia 2

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Abstract

Aim: The aim of this study was to evaluate the clinical performance of the Amplicor HPV test, which detects 13 high-risk human papillomaviruses (HR-HPV), and to determine the association between consistent HR-HPV infection and progression of cervical intraepithelial neoplasia (CIN) 2 to CIN3.

Material and Methods: This multi-institutional prospective study enrolled 122 women diagnosed with CIN2 by central pathological review. Subjects were tested at study entry and every 6 months over a 24-month period by cytology, Amplicor HPV test and colposcopy. Central pathological review was performed at the end of the study or if CIN progression was suspected.

Results: Ninety-three of the 122 participants completed all tests in the study and were included in the analysis. HR-HPV was detected in 87/93 (93.5%) participants at study entry. Twenty-four of the 87 HR-HPV-positive participants progressed to ≥CIN3, compared with none of the six participants who were HR-HPV-negative at study entry. The positive predictive value, negative predictive value, sensitivity and specificity of the Amplicor HPV test at study entry for predicting ≥CIN3 progression were 27.6%, 100%, 100% and 8.7%, respectively. Sixty-two participants were HR-HPV-positive from study entry through to study completion, 24 of whom progressed to ≥CIN3. None of 31 participants without continuous HR-HPV detection progressed to ≥CIN3. For continuous HR-HPV detection, the positive predictive value, negative predictive value, sensitivity and specificity of the Amplicor HPV test were 38.7%, 100%, 100% and 44.9%, respectively.

Conclusions: All participants who progressed to ≥CIN3 were continuously HR-HPV-positive. The Amplicor HPV test thus demonstrated a good performance for predicting CIN3 progression.

Key words: Amplicor HPV test, cervical intraepithelial neoplasia progression, cervical intraepithelial neoplasia regression, continuous infection, high-risk human papillomavirus.

Introduction

Cervical cancer is the second most common gynecologic malignancy worldwide.[1] Human papillomavirus (HPV) is the most common sexually transmitted infection, and a subset of HPV genotypes is now recognized as a single, necessary cause of cervical cancer.[2] Between 13 and 16 HPV genotypes are currently classified as

Received: July 9 2012.
Accepted: December 28 2012.
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carcinogenic or high-risk (HR) HPV,[3,4] and their persistent infection has a critical causative function in the development of cervical intraepithelial neoplasia (CIN) and progression of precancerous CIN3 to cervical cancer.[5] Accordingly, HPV DNA testing has become an important part of cervical cancer screening programs.

However, while the majority of sexually active adults will be exposed to HR-HPV during their lifetime, only a small percentage develop CIN3 or carcinoma; the majority of women infected with HR-HPV do not develop CIN3 and eliminate the HR-HPV by an active immune response. Moreover, this immunity also provides lasting protection from re-infection with the same HR-HPV genotype.

HR-HPV testing is more sensitive than cytology for detecting CIN2 or worse pathology in a single screening interval, but shows lower specificity than cytology for CIN3 in a single screening interval. Current cervical cancer screening in Japan is therefore based primarily on cytology to identify women at risk of having or developing CIN3.

CIN3 has been reported to have a high rate of progression to invasive cervical cancer[6] and therefore it is important to investigate the process of progression from CIN2 to ≥CIN3. However, few prospective studies have addressed HR-HPV infection and the timeframe associated with this progression.[7,8] We conducted a multi-institutional 24-month prospective CIN2 cohort study in Japan designed to examine the development of ≥CIN3 in a cohort of women with histologically confirmed central pathological review (CPR)-diagnosed CIN2.

Cervical cytology is the principal test used to screen for cervical cancer, but it has the disadvantage of low sensitivity for detecting CIN2 or CIN3 in one screening interval.[9] We prospectively evaluated HR-HPV testing using the Amplicor HPV test in combination with cytology for predicting CIN2 progression outcomes over a 2-year period.

Methods

Study design

Two hundred women diagnosed with CIN2 at 24 hospitals geographically distributed throughout Japan were recruited from January 2007 to May 2008 and their biopsies were subjected to CPR for study enrollment. CPR was performed blindly by two pathologists from the Cancer Institute Hospital of the Japanese Foundation for Cancer Research, Department of Pathology. Disagreements between their review results were resolved by discussion. Thirty-eight women with CIN1, 28 women with CIN3 and 12 women with other diagnoses were excluded from the study, and 122 women with CIN2 were finally enrolled as participants. All participants entered the study only after voluntarily signing informed consent. This study was approved by the Institutional Review Boards of each participating hospital. The inclusion criteria for the study, in addition to confirmed CIN2 by CPR, were age 20–50 years and no previous history of cervical abnormality. A cervical sample collected by liquid-based cytology (LBC), HR-HPV test (Amplicor HPV test), and colposcopy were performed at study entry and again at each 6-month visit throughout the 24 months of follow-up. When progression was suspected, cervical biopsy and CPR diagnosis were performed. All participants diagnosed as CIN3 by CPR exited the study for treatment and were scored as CIN3 progression.

At the end of follow-up, 29 participants were excluded from the final analysis for the following reasons: eight participants received treatment before completion of the study period, five became pregnant, three moved out of the study range, four were lost to follow-up, four found it difficult to get to the hospital, and five exited for other reasons. At the end of the 2-year follow-up period, study results from 93 participants were available for analysis.

Cervical sample collection

Cervical samples were taken using a Cervix brush (Roven Medical Devices). The brush with the specimen was suspended in SurePath Preservative Fluid (TriPath Imaging) and stored at 2–8°C. The resulting sample solution was subjected to HR-HPV testing and LBC within 2 weeks.

CPR and LBC

Papanicolaou-stained sample slides were prepared from the SurePath sample using the Autocyte Preparation System. The slides were screened by cytotechnologists and then diagnosed by medical specialists according to the classification of the Japan Association of Obstetricians and Gynecologists, which modified the Papanicolaou classification.[10] This classification system was the most popular cytological assessment system in Japan at the start of this study.

CPR was performed according to the World Health Organization histological typing guidelines using samples acquired by colposcopy-directed punch biopsy at each hospital.

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HPV detection

DNA was extracted from 250 μL of the SurePath sample solution and subjected to HR-HPV testing. HR-HPV tests were performed using the Amplicor HPV test, according to the manufacturer's instructions. The Amplicor HPV test involves PCR amplification of target DNA, followed by hybridization in a microwell plate with probes that bind to 13 HR-HPV genotypes, that is, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Amplified DNA containing biotin bound to immobilized probes was then detected in a colorimetric reaction. An optical density ≥0.2 indicated that the sample was positive for one or more of the 13 HR-HPV genotypes.

Statistical methods

The *t*-test was used to compare ages between the participants who progressed and those who did not progress to ≥CIN3.

Results

Of the 122 participants enrolled in this study, 29 participants were withdrawn and 93 participants completed the study with multiple HR-HPV test results and biopsy outcomes available for analysis. The mean age of the participants who completed the study was 37.1 ± 6.4 years.

Table 1 shows the results of the Amplicor HPV tests at study entry and the CPR diagnoses at study completion. At the entry-point, 87 of 93 (93.5%) participants tested positive for HR-HPV by the Amplicor HPV test. During the 2-year study period, 24/93 (25.8%) participants progressed to ≥CIN3. CPR outcomes at study completion indicated that CIN2 pathology persisted as CIN2 in 14/93 (15.1%) participants and regressed to CIN1 in 55/93 (59.1%) participants. Age was not a significant factor in predicting CIN2 progression to CIN3 or regression to CIN1; the mean age for CIN3 progressors was 36.5 ± 6.4 years, compared with 37.2 ± 6.5 years for non-progressors. The 24 participants who progressed to ≥CIN3 were all HR-HPV-positive at the study entry-point, whereas no HR-HPV-negative participants progressed to ≥CIN3 within 2 years. A single negative HR-HPV test at the entry-point was highly associated with regression, and the CIN2 pathology in 5/6 participants who tested HR-HPV-negative at the entry-point were confirmed by CPR to regress to CIN1 during the course of the study. However, regression was also observed in some participants who tested HR-HPV-positive at baseline. Of the participants with HR-HPV at the entry-point, 57.5% (50/87) were confirmed by CPR to regress to CIN1 over the 2-year study period. The 24 participants who progressed to ≥CIN3 were all still HR-HPV-positive at 6 months, while none of 11 participants who became HR-HPV-negative at 6 months progressed to ≥CIN3 within 2 years (data not shown).

Table 2 shows the results of continuous Amplicor HPV testing and CPR at the study completion point.

Table 1 Results of Amplicor HPV testing at entry-point and CPR at completion point

Amplicor HPV test result at entry-point	Total	CPR result at completion point			
		≥CIN3	≤CIN2	CIN2	CIN1
	n	*n*	*n*	*n*	*n*
HR-HPV-positive	87	24	63	13	50
HR-HPV-negative	6	0	6	1	5
Total	93	24	69	14	55

CIN, cervical intraepithelial neoplasia; CPR, central pathological review; HPV, human papillomavirus; HR, high-risk.

Table 2 Results of continuous Amplicor HPV testing and CPR at completion point

Continuous HR-HPV infection result with Amplicor HPV test	Total	CPR result at completion point			
		≥CIN3	≤CIN2	CIN2	CIN1
	n	*n*	*n*	*n*	*n*
Continuous HR-HPV-positive	62	24	38	13	25
Not continuous HR-HPV-positive	31	0	31	1	30
Total	93	24	69	14	55

CIN, cervical intraepithelial neoplasia; CPR, central pathological review; HPV, human papillomavirus; HR, high-risk.

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Table 3 Performance characteristics of Amplicor HPV test for prediction of progression to ≥CIN3

	Amplicor HPV test at entry-point	Amplicor HPV test at 6 months	Continuous Amplicor HPV testing
PPV (95%CI)	27.6 (18.5–38.2)	29.3 (19.7–40.4)	38.7 (26.6–51.9)
NPV (95%CI)	100 (54.1–100)	100 (71.5–100)	100 (88.8–100)
Sensitivity (95%CI)	100 (85.7–100)	100 (85.7–100)	100 (85.7–100)
Specificity (95%CI)	8.7 (3.3–18.0)	15.9 (8.2–26.7)	44.9 (32.9–57.4)

CI, confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; NPV, negative predictive value; PPV, positive predictive value.

Table 4 Results of LBC at 6 months and CPR at completion point

LBC result at 6 months	Total	CPR result at completion point			
		≥CIN3	≤CIN2	CIN2	CIN1
	n	*n*	*n*	*n*	*n*
≥Class IIIb†	27	14	13	3	10
≤Class IIIa‡	65	10	55	11	44
Total	92§	24	68	14	55

†Severe dysplasia. ‡Mild and moderate dysplasia. §No LBC data for one participant. CIN, cervical intraepithelial neoplasia; CPR, central pathological review; LBC, liquid-based cytology.

Twenty-five of 87 participants who were HR-HPV-positive at the entry-point became HR-HPV-negative within 2 years, and a total of 31 (33.3%) participants were not continuously HR-HPV-positive for the duration of the study. None of the 31 participants who were not continuously HR-HPV-positive progressed to ≥CIN3, and 96.7% (30/31) of them regressed to CIN1 within 2 years. A total of 62 (66.7%) participants were continuously HR-HPV-positive from study entry to completion, including all participants who progressed to ≥CIN3. Thus, continuous HR-HPV-positivity appears to be necessary not only for progression to ≥CIN3, but also for persistence of CIN2.

Table 3 shows the performance characteristics of the Amplicor HPV test. The positive predictive values (PPV), negative predictive values (NPV), sensitivities and specificities of the Amplicor HPV test at the entry-point, at 6 months, and on continuous testing from study entry to completion are summarized. Amplicor HPV tests showed good performance in terms of NPV and sensitivity, which were 100% in all cases. This means that participants who were HR-HPV-negative at any point did not progress to ≥CIN3 within 2 years, and all participants who did progress to ≥CIN3 were HR-HPV-positive at all points. When HR-HPV testing at only the entry-point was analyzed, the PPV and specificity of the Amplicor HPV test were low (27.6 and 8.7%, respectively). In contrast, repeated HR-HPV testing and continuous investigation of HR-HPV infection increased the PPV and specificity to 38.7% and 44.9%, respectively.

In this study, LBC was performed every 6 months, in addition to the Amplicor HPV test. The LBC sample taken at study entry was collected close to the time of biopsy taken for pathology at each hospital, and the cytological outcome could thus have been influenced by the biopsy collection. The cytological results from LBC samples collected at 6 months were therefore evaluated, instead of the LBC results at the entry-point. Table 4 shows the results of LBC at 6 months and CPR at the completion point. There were no LBC results at 6 months for one participant, and the total number in Table 4 is therefore 92. Twenty-seven participants were diagnosed by LBC with ≥class IIIb, and 65 participants were diagnosed with ≤class IIIa at 6 months, based on the cytological classification of the Japan Association of Obstetricians and Gynecologists. This Japanese classification system divides class III into IIIa and IIIb, defining cases with mild and moderate dysplasia, and with severe dysplasia, respectively. Fourteen (51.8%) out of 27 participants with ≥class IIIb cytology and 10 (15.4%) out of 65 participants with ≤class IIIa cytology progressed to ≥CIN3 within 2 years. Ten (90.0%) of the 11 HR-HPV-negative participants at 6 months and 44 (67.7%) of the 65 ≤class IIIa participants regressed to CIN1 within 2 years (Table 4). LBC cytology thus

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Table 5 Results of Amplicor HPV tests at 6 months and CPR at completion point in ≥class IIIb and ≤class IIIa participants by LBC at 6 months

Amplicor HPV test result at 6 months	≤class IIIa			≥class IIIb		
	Total	CPR result at completion point		Total	CPR result at completion point	
		≥CIN3	≤CIN2		≥CIN3	≤CIN2
	n	*n*	*n*	*n*	*n*	*n*
HR-HPV-positive	60	10	50	21	14	7
HR-HPV-negative	5	0	5	6	0	6
Total	65	10	55	27	14	13

CIN, cervical intraepithelial neoplasia; CPR, central pathological review; HPV, human papillomavirus; HR, high-risk; LBC, liquid-based cytology.

Table 6 Performance characteristics of LBC alone, Amplicor HPV test alone, and Amplicor HPV in combination with LBC for prediction of progression to ≥CIN3

	LBC alone	Amplicor HPV test alone	≥class IIIb and Amplicor HPV test
PPV (95%CI)	51.9 (31.9–71.3)	29.6 (12.9–51.5)	66.7 (43.0–85.4)
NPV (95%CI)	84.6 (73.5–92.4)	100 (71.5–100)	100 (76.8–100)
Sensitivity (95%CI)	58.3 (36.6–77.9)	100 (85.7–100)	100 (54.1–100)
Specificity (95%CI)	80.9 (69.5–89.4)	16.2 (4.5–36.8)	46.2 (19.2–74.9)

CI, confidence interval; CIN, cervical intraepithelial neoplasia; LBC, liquid-based cytology; NPV, negative predictive value; PPV, positive predictive value.

seems to predict regression less accurately than the Amplicor HPV test.

Table 5 shows the results of Amplicor HPV tests at 6 months and CPR at the completion point in two groups divided on the basis of their LBC results at 6 months. Ten (15.4%) of the 65 participants with ≤class IIIa progressed to ≥CIN3 in HR-HPV-positive cases, whereas six (22.2%) of the 27 participants with ≥class IIIb did not progress in HR-HPV-negative cases.

Table 6 shows the performance characteristics of LBC at 6 months, the Amplicor HPV test at 6 months, and the Amplicor HPV test in combination with LBC. The results of the Amplicor HPV test at 6 months were recalculated based on a total number of 92, because one participant had no LBC data at 6 months. Compared with the Amplicor HPV test at 6 months alone, the LBC results showed a higher PPV (29.6 vs 51.9%) and specificity (16.2 vs 80.9%), but a lower NPV (100 vs 84.6%) and sensitivity (100 vs 58.3%) for the prediction of progression.

In the case of ≥class IIIb, the PPV increased from 51.9% to 66.7% for LBC alone, and from 29.6% for the Amplicor HPV test alone. The participants who progressed to ≥CIN3 could be predicted more accurately by a combination of LBC and the Amplicor HPV test. The NPV and sensitivity increased from 84.6% and 58.3%, respectively, for LBC alone, to 100% for LBC in combination with the Amplicor HPV test. Participants giving false-negative results with LBC could be detected when LBC was combined with the Amplicor HPV test. The low specificity of the Amplicor HPV test increased from 16.2% to 46.2% in combination with LBC.

Discussion

Cervical cancer is now recognized to be caused by persistent infection with carcinogenic HR-HPV, which develop primarily as precancerous CIN lesions and then progress to invasive cancer.[2,3,5,6] It is therefore desirable to screen and treat patients at the CIN stage, before progression to cancer. The 2006 consensus guidelines of the American Society for Colposcopy and Cervical Pathology (ASCCP) recommend ablation and excision for CIN2 and 3, with a high evidence level.[11]

In this prospective cohort study, we recruited women diagnosed with CIN2 by pathologists at each hospital, and only women further diagnosed with CIN2 according to CPR were enrolled. The CPR was performed by two pathologists, as in a recent report from Japan.[8] The diagnosis of CIN2 has been reported to be less reproducible than CIN3.[12] In this study, however, CIN2 was diagnosed by at least three pathologists (a pathologist from the hospital in

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addition to two CPR pathologists) making the chance of misdiagnosis of CIN2 at study entry unlikely.

In this study, 122 women with CIN2 were enrolled and cervical samples were analyzed every 6 months for cytology and by the Amplicor HPV test, which detects 13 HR-HPV, during a 2-year follow-up period. Twenty-nine participants exited the study for various reasons, and 93 participants were thus finally analyzed.

The HR-HPV infection rate at the entry-point was 93.5%. This was higher than in previous studies in Japan, which reported that 54 (80.6%) out of 67 participants with CIN2, 231 (79.4%) of 291 participants with CIN 2 and 3, and 60 (89.5%) of 67 participants with CIN2 were HR-HPV-positive.[7,8,13] The progression rate of 25.8% in 2 years was also high compared with previous studies, which found that 24.2% of 91 participants with CIN2 progressed to CIN3 in 5 years[8] and 15.4% of 71 participants with CIN2 progressed to CIN3 in 3.1–57 months.[7] The high infection and progression rates in this study may reflect the fact that CPR diagnosed CIN2 very strictly with a special criterion[14] at the entry-point.

The 24 participants who progressed to ≥CIN3 were all HR-HPV-positive at the entry-point, while none of the participants who were HR-HPV-negative at the entry-point progressed to ≥CIN3. The NPV and sensitivity of the Amplicor HPV test performed at the entry-point were thus both 100%; however, its PPV and specificity were low. These results are consistent with previous studies.[8,15,16]

When the Amplicor HPV test was performed periodically, its PPV and specificity increased, and its NPV and sensitivity remained at 100%. Thus the 24 participants who progressed to ≥CIN3 were all continuously HR-HPV-positive throughout the study, while none of the 31 participants without continuous HR-HPV infection progressed to ≥CIN3. This shows that continuous HR-HPV infection is important for CIN progression.

From the viewpoint of regression, five (83.3%) of six participants without HR-HPV infection at the entry-point and 30 (96.8%) of 31 participants without continuous HR-HPV infection regressed to CIN1 within 2 years, indicating that the Amplicor HPV test could predict CIN regression in negative cases. The Amplicor HPV test is thus useful for predicting not only CIN progression, but also CIN regression.

Cytological tests have been reported to be insufficient for cervical cancer screening, mainly because of their low PPV and sensitivity.[9] When ≤class IIIa and ≥class IIIb cases were divided into HR-HPV-positive and -negative cases, the LBC at 6 months showed an NPV of 84.6% and a sensitivity of 58.3%. The Amplicor HPV test in combination with LBC compensated for the disadvantage of LBC and identified the false negatives and false positives picked out by LBC alone. The combination of the Amplicor HPV test and LBC increased the relatively low PPV and specificity. These results thus confirmed the practical usefulness of HR-HPV testing for triage of LBC-diagnosed participants with CIN2, as previously reported.[9,15,16]

The number of patients with CIN3 has increased dramatically in Japan, from 2679 in 2002 to 7116 in 2009,[17,18] suggesting that many women might be newly diagnosed with CIN2 every year. Management at the CIN2 stage is thus an urgent issue in Japan, and the detection of persistent HR-HPV infection appears to be essential for adequate treatment of CIN2.

In conclusion, the results of this study suggest that the Amplicor HPV test is useful for the prediction of not only CIN progression, but also CIN regression. In addition, the Amplicor HPV test in combination with LBC can increase the detection of high-risk cases and exclude low-risk cases in participants with CIN2.

Acknowledgments

The authors thank Roche Diagnostics K.K., Tokyo, Japan for financial and practical support in this study. They would also like to thank Osaka City General Hospital, Fujita Health University Hospital, Keio University Hospital, Jikei University Hospital, Tohoku University Hospital, University of Ryukyu Hospital, Nagoya University Hospital, Kurume University Hospital, Niigata University Medical & Dental Hospital, Teikyo University Hospital, Kagoshima City Hospital, Hokkaido University Hospital, Iwate Medical University Hospital, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tottori University Hospital, Kitasato University Hospital and Sapporo Medical University Hospital for patient enrollment.

Disclosure

H. Tanaka and K. Watanabe are employees of Roche Diagnostics K.K. The other authors declare that they have no conflicts of interest.

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Published OnlineFirst January 22, 2013; DOI:10.1158/1078-0432.CCR-12-2199

Clinical Cancer Research

PD-L1 on Tumor Cells Is Induced in Ascites and Promotes Peritoneal Dissemination of Ovarian Cancer through CTL Dysfunction

Kaoru Abiko, Masaki Mandai, Junzo Hamanishi, et al.

Clin Cancer Res 2013;19:1363-1374. Published OnlineFirst January 22, 2013.

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Human Cancer Biology

Clinical Cancer Research

PD-L1 on Tumor Cells Is Induced in Ascites and Promotes Peritoneal Dissemination of Ovarian Cancer through CTL Dysfunction

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Abstract

Purpose: Ovarian cancer often progresses by disseminating to the peritoneal cavity, but how the tumor cells evade host immunity during this process is poorly understood. Programmed cell death 1 ligand 1 (PD-L1) is known to suppress immune system and to be expressed in cancer cells. The purpose of this study is to elucidate the function of PD-L1 in peritoneal dissemination.

Experimental Design: Ovarian cancer cases were studied by microarray and immunohistochemistry. PD-L1 expression in mouse ovarian cancer cell line in various conditions was assessed by flow cytometry. PD-L1–overexpression cell line and PD-L1–depleted cell line were generated, and cytolysis by CTLs was analyzed, and alterations in CTLs were studied by means of timelapse and microarray. These cell lines were injected intraperitoneally to syngeneic immunocompetent mice.

Results: Microarray and immunohistochemistry in human ovarian cancer revealed significant correlation between PD-L1 expression and peritoneal positive cytology. PD-L1 expression in mouse ovarian cancer cells was induced upon encountering lymphocytes in the course of peritoneal spread *in vivo* and coculture with lymphocytes *in vitro*. Tumor cell lysis by CTLs was attenuated when PD-L1 was overexpressed and promoted when it was silenced. PD-L1 overexpression inhibited gathering and degranulation of CTLs. Gene expression profile of CTLs caused by PD-L1–overexpressing ovarian cancer was associated with CTLs exhaustion. In mouse models, PD-L1 depletion resulted in inhibited tumor growth in the peritoneal cavity and prolonged survival.

Conclusion: PD-L1 expression in tumor cell promotes peritoneal dissemination by repressing CTL function. PD-L1–targeted therapy is a promising strategy for preventing and treating peritoneal dissemination. *Clin Cancer Res; 19(6); 1363–74. ©2012 AACR.*

Introduction

Ovarian cancer is the most lethal disease among gynecologic malignancies. Unlike other epithelial tumors, peritoneal dissemination is the most common mechanism of disease progression in ovarian cancer, and up to 70% of cases present with massive malignant ascites and peritoneal implants (1). Control of dissemination seems to be the most important strategy in controlling ovarian cancer because the median overall survival and progression-free survival are 81.1 and 35.0 months, respectively, if macroscopically complete surgical resection of the disseminated tumors is achieved in Federation Internationale des Gynaecologistes et Obstetristes (FIGO) stage IIIc cancers, whereas these measures are only 34.2 and 14.5 months, respectively, if the disseminated tumor remains after the initial surgery (2). The peritoneal cavity is also the most frequent site of recurrence, and most patients who undergo intraperitoneal recurrence die from this disease (3).

At least 3 steps, cell detachment, immune evasion, and implantation, are required for dissemination. Various molecules expressed by cancer cells have been reported to be involved in these steps (4). In cell detachment, molecules that cause epithelial-to-mesenchymal transition, such as TGF-β or Snail, have an important role (5–7). In implantation, extracellular matrix proteins and VEGF are thought to be important (8). In addition, cancer cells potentially must escape from attack by the immune cells that they encounter in the peritoneal cavity. Immune evasion during peritoneal dissemination is the most enigmatic step. Lymphocytes isolated from malignant ascites have shown

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-12-2199

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Translational Relevance

Immune evasion is one of the emerging hallmarks of cancer, though most of its mechanisms remain unveiled. Ovarian cancer often progresses by disseminating to the peritoneum, but how the tumor cells evade host immunity during this process is poorly understood. In this study, we have shown that ovarian cancer cells express programmed cell death 1 ligand 1 (PD-L1) upon encountering lymphocytes in the peritoneal cavity, and as a consequence, inhibit CTL function, escape from CTLs, and disseminate into the peritoneal cavity. Depleting PD-L1 expression in tumor cells resulted in inhibited tumor growth in the peritoneal cavity and prolonged survival of the mice. These data show for the first time that host–tumor immunity, especially tumor immune escape mechanisms, has a pivotal role in peritoneal dissemination. Our data suggest that restoring immune function by inhibiting immune-suppressive factors such as PD-L1 is a promising strategy for controlling the peritoneal dissemination of malignant tumors, including ovarian cancer.

tumoricidal activity (9), but the mechanisms by which the tumor cells evade these cells are not clearly understood. Secretion of Fas ligands by ovarian cancer cells (10), the recruitment of regulatory T cells (11), and the T-cell suppressor cytokine phenotype of monocytes and macrophages (12) have been reported to be included in this step, but the precise mechanism of tumor evasion from immune cells remains unclear.

Recent studies have added immune evasion as one of the important hallmarks of cancer (13). Restoring immune function in cancer microenvironment has immense potential for a new cancer therapy (14). We have attempted to elucidate the mechanism of immune escape in ovarian cancer and reported that in the ovarian cancer microenvironment, molecules such as ULBP2 (NKG2D ligand), COX-1, COX-2, and programmed cell death 1 ligand 1 (PD-L1) or the combined expression of these molecules are related to limited infiltration by lymphocytes and an unfavorable prognosis (15–18). PD-L1 (also known as B7-H1 or CD274) is a coregulatory molecule that is expressed on the surface of various types of cells, including immune cells and epithelial cells. By binding to its receptor PD-1 on lymphocytes, it generates an inhibitory signal toward the T-cell receptor (TCR)-mediated activation of lymphocytes (19, 20). We have reported that PD-L1 expression in tumor cells is an independent unfavorable prognostic factor in human ovarian cancer (15), and that PD-L1 expression showed the closest relation to unfavorable prognosis among other immunosuppressive molecules that we have tested (18). These data suggest that PD-L1 has a role in the clinical course of ovarian cancer by affecting the local immune microenvironment and that PD-L1/PD-1 signal could be a potential therapeutic target. Actually, a recent clinical trial of systemic administration of anti-PD-1 or anti-PD-L1 antibody showed a promising clinical effect in several solid tumors (21–23). However, the role of PD-L1 or the precise mechanism of immune escape in the process of peritoneal dissemination is poorly understood.

The aim of this study was to investigate the mechanism by which PD-L1 on cancer cells in ascites enables immune evasion during peritoneal dissemination, by using both clinical samples and mouse models.

Materials and Methods

Survival analysis of ovarian cancer patients

A total of 65 patients with epithelial ovarian cancer (KOV-IH-65), who underwent primary operation at Kyoto University Hospital (Kyoto, Japan) between 1997 and 2002 and the outcome and peritoneal cytology was evaluable from the chart was included in the study under the approval of the Kyoto University Graduate School and Faculty of Medicine Ethics Committee. Ascites or the peritoneal wash fluid was collected at operation and served for pathologic diagnosis. Patient characteristics are listed in Supplementary Table S1.

Microarray profiling of ovarian cancer tissues

Ovarian cancer specimens were obtained from 64 patients (KOV-MA-64), who underwent primary surgery for epithelial ovarian cancer at Kyoto University Hospital between 1997 and 2011. Ten patients in KOV-IH-65 were included in KOV-MA-64. All tissue specimens were collected under written consent approved by the Facility Ethical Committee. Patient characteristics are listed in Supplementary Table S1. Samples were selected to have more than 70% tumor cell nuclei and less than 20% necrosis. Total RNA expression was analyzed on Human Genome U133 Plus 2.0 Array (Affymetrix). Robust multiarray average (RMA) normalization was conducted using R (R: a language and environment for statistical computing; http://www.R-project.org.). Probes showing expression value more than 5.0 in at least one of the samples and SD more than 0.2 across all the samples were selected, and *t* test was conducted between cytology-positive and -negative groups. Enrichment for Gene Ontology terms was analyzed using GOEAST software (http://omicslab.genetics.ac.cn/GOEAST/; ref. 24) for the set of probes highly expressed in cytology-positive or -negative groups, respectively ($P < 0.05$). A publicly accessible gene set of IFN-γ-upregulated genes was downloaded (http://www.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=SANA_RESPONSE_TO_IFNG_UP; ref. 25). Gene set enrichment analysis (GSEA) for positive ascites cytology and negative cytology was conducted using GSEA software (http://www.broadinstitute.org/gsea/downloads.jsp).

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of ovarian cancer were obtained from KOV-IH-65 patients under written consent as earlier. Immunohistochemical staining for PD-L1 was conducted using a PD-L1 antibody as previously described (15, 18). PD-L1 expression was analyzed by 2

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independent gynecologic pathologists without any prior information about the clinical history of the patients, and the samples were categorized into a positive expression group (equal to or stronger than the positive control) and a negative expression group (weaker than the positive control) based on the intensity of the staining. Placenta was used as positive control. Samples with staining in less than 50% of tumor cells was considered negative.

Animals

Female C57BL/6 (B6) and B6C3F1 and C.B-17/lcr-scid/scidJcl [severe combined immunodeficient mice (SCID)] mice were purchased from CLEA Japan. OT-1 mice and CAG-GFP mice were purchased from the Jackson Laboratory and were interbred to generate OT-1-GFP mice. Animal experiments were approved by the Kyoto University Animal Research Committee, and animals were maintained under specific pathogen-free conditions. To evaluate the effect of PD-L1 on the survival and progression of peritoneal dissemination and ascites formation, HM-1 cells (1×10^6) or ID8 cells (5×10^6) were injected into the abdominal cavity. The body weight gain was calculated every other day. Mice were euthanized before reaching the moribund state.

Cell lines

The ID8 mouse ovarian cancer cell line (26, 27) was kindly provided by Dr. Margit Maria Janát-Amsbury (Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Baylor College of Medicine, Houston, TX; ref 27). The cells were maintained in RPMI-1640 medium (Nacalai Tesque) supplemented with 10% FBS (v/v; Biowest) and penicillin–streptomycin (Nacalai Tesque). The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line was purchased from RIKEN BioResource Center and cultured as previously described (7). Human ovarian cancer cell lines were cultured as described previously (28). The ID8-GFP cells and HM1-GFP cells were generated by retroviral transfection as described previously (29).

The PD-L1–overexpressing cell lines, ID8-pdl1 and HM1-pdl1, were generated by lentiviral transfection of ViraPower pLenti6/V5-DEST Gateway Vector (Invitrogen) carrying mouse PD-L1 cDNA. Full-sequenced cDNA was purchased from OpenBiosystems and amplified by PCR using the following primers:

Forward; CACCAACATGAGGATATTTGCTGG
Reverse; TCAACACTGCTTACGTCTCC

Expression vector was generated using pENTR Directional TOPO Cloning Kit (Invitrogen).

The PD-L1–depleted cell lines, ID8-Mirpdl1 and HM1-Mirpdl1, were generated using the BLOCK-iT HiPerform Lentiviral Pol II miR RNAi Expression System with EmGFP (Invitrogen) according to the protocol provided by the manufacturer. Briefly, double-stranded oligos were generated from designed single-stranded DNA oligos listed later, and cloned into pcDNA™6.2-GW/EmGP-miR expression vector. Then, it was linearized and BP/LR Reaction was conducted using pDONR™221 vector and pLenti6.4/R4R2/V5-DEST and pENTR™5′ promoter clone to generate Lentiviral expression clone. The sequence of the miR DNA oligos used for PD-L1 depletion is as follows:

Top strand oligo;
TGCTGTTCAACGCCACATTTCTCCACGTTTTGGCCACT-GACTGACGTGGAGAAGTGGCGTTGAA
Bottom strand oligo;
CCTGTTCAACGCCACTTCTCCACGTCAGTCAGTGGCCA-AAACGTGGAGAAATGTGGCGTTGAAC

Sequence control cell lines (ID8-control and HM1-control) were generated using a nonsilencing miR oligo provided by the manufacturer.

A concentration of 20 ng/mL recombinant human IFN-γ (R&D Systems) or recombinant mouse IFN-γ (PeproTech) was added to the culture medium for 24 hours before analysis for IFN-γ stimulation. For the other recombinant mouse cytokines, 200 ng/mL interleukin (IL)-2 (eBioscience) or 20 ng/mL IL-6 (R&D Systems), TGF-β (PeproTech), IL-10 (PeproTech), or TNF-α (PeproTech) was added to the culture medium for 24 hours before analysis.

Flow cytometry

Cultured cells were harvested and incubated with phycoerythrin (PE)-conjugated PD-L1 (mouse clone MIH5, human clone MIH1; BD Biosciences) or a matched isotype control (BD Biosciences) at 4°C for 30 minutes, washed twice, and analyzed using a FACSCalibur cytometer (Beckton Dickinson). The results were analyzed using CellQuest Pro software.

Analysis of PD-L1 expression on tumor cells in ascites

Mice were challenged with an intraperitoneal injection of the GFP-labeled cell lines. Mice with ascites formations were sacrificed and the ascites were collected. After briefly centrifuging, red blood cells were lysed, and the remaining cells were washed twice, incubated with antibodies, and analyzed by flow cytometry as mentioned previously. 7-AAD Staining Solution (BD Biosciences) was added 10 minutes before analysis to gate out nonviable cells. GFP-positive and 7-amino-actinomycin D–negative gated cells were analyzed as ascites tumor cells.

$CD8^+$ T lymphocyte collection from ascites

Mouse ascites cells were collected and washed with PBS supplemented with 2% FBS. $CD8^+$ T lymphocyte was collected by magnetic separation using mouse CD8a MicroBeads (Miltenyi Biotec).

Detection of intracellular IFN-γ in mouse lymphocytes

For intracellular IFN-γ staining, BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and PE-conjugated anti-mouse IFN-γ antibody (BD Biosciences) were used. A matched isotype control was used to determine IFN-γ–negative quadrant. PerCP-conjugated anti-mouse CD3e antibody (BD Biosciences), Alexa Fluor 647-

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conjugated anti-mouse CD8a antibody (BD Biosciences), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 antibody were used to gate lymphocytes and $CD4^+$ or $CD8^+$ cells.

Multiplexed bead assay for cytokines in ascites

BD CBA Mouse Th1/Th2/Th17 Kits (BD Biosciences) was used according to the manufacturer's protocol. Concentrations of each cytokines were calculated using BD Cytometric Bead Array Software version 1.4 (BD Biosciences).

Proliferation assay

A water soluble tetrazolium-8 assay using Cell Count Reagent SF (Nacalai Tesque) was conducted according to the manufacturer's protocol, and the proliferation rate for each cell line was calculated and plotted.

Activation of CTLs

B6 splenocytes underwent T-cell depletion using CD90.2 Microbeads (Miltenyi Biotec) and were incubated with 10 μg/mL $OVA_{257-264}$ peptide (SIINFEKL, Bachem Bioscience) at 37°C for 1 hour. Then they were coincubated with $CD8^+$ cells that were isolated from female OT-1-GFP mice using $CD8a^+$ T Cell Isolation Kit II (Miltenyi Biotec) for 4 to 6 days. Subsequently, the CTLs were collected by CD8a MicroBeads (Miltenyi Biotec) and were used for further experiments. RPMI-1640 medium supplemented with 10% FBS, 50 μmol/L 2-mercaptoethanol (Nacalai Tesque), 2 mmol/L L-glutamine (Invitrogen), and penicillin–streptomycin (Nacalai Tesque) was used for lymphocyte cultures.

Cytotoxicity assay

As target cells, ID8 cells were loaded with 10 μg/mL $OVA_{257-264}$ peptide (Bachem Bioscience) at 37°C for 1 hour. As effectors, activated OT-1 $CD8^+$ CTLs were prepared as described earlier. The target cells were cocultured with the effector cells at various E/T (effector-to-target) ratios. After 5 hours of incubation, the levels of lactate dehydrogenase in the culture supernatant were determined using the cytotoxicity detection kit CytoTox96 (Promega). We used 0.9% Triton X to determine maximum target cell lysis. Percentage lysis was calculated according to a modified standard formula:

$$(OD_{experimental} - OD_{spontaneous\ targets} - OD_{spontaneous\ effectors})/(OD_{maximum} - OD_{spontaneous\ targets}) \times 100.$$

CD107a expression assay

After 4 hours of coincubation of target cells and OT-1-GFP mouse CTLs at an E/T ratio of 30, the cells were incubated with an Alexa Fluor 647–conjugated anti-CD107a antibody (BioLegend) and were washed twice and analyzed by flow cytometry. GFP-positive cells were gated as OT-1-GFP mouse CTLs.

Time-lapse photography of CTLs attacking target cells

CTLs from OT-1-GFP mouse were activated as described earlier. A total of 3×10^6/mL CTLs were mixed with 1×10^5/mL ID8-control or ID8-pdl1 cells loaded with OVA peptide and observed under a laser microscope (Olympus TH4-100) at magnification of ×200. Images of GFP-positive cells were acquired every 2 minutes for total of 68 times (=136 minutes) using DP71-MetaMorph system. Time-lapse video was made from these images (10 frames/s) using MetaMorph software (Molecular Devices).

Microarray profiling of CTLs

OT-1-GFP-mouse CTLs were collected from 4 mice (mouse A to D) and activated as described earlier. CTLs from mouse A to D were divided into 2 groups and coincubated with ID8-pdl1 (PD-L1 group) or ID8-Mirpdl1 (Mir group) for 4 hours at an E/T ratio of 30. Then, the activated CTLs were collected by magnetic separation using CD8a Microbeads (Miltenyi Biotec). From these 8 samples of CTLs, whole RNA was extracted with RNAeasy Kit (Qiagen) and hybridized to Affymetrix Mouse Genome430 2.0 Array as previously described (5). RMA normalization was conducted as described earlier. Gene sets for CTL_PD-L1_UP (high in PD-L1 group) and CTL_PD-L1_DN (high in Mir group) were generated using paired t test between the 2 groups ($P < 0.01$). GSE24026 dataset, which analyzed downstream of PD-1 signaling (30), was downloaded from Gene Expression Omnibus (GEO) DataSets (http://www.ncbi.nlm.nih.gov/gds) to analyze the association of PD-1 signaling with our experiments.

Statistics

For the analysis of immunohistochemistry, Fisher exact test and the χ^2 test were used to analyze the associations between PD-L1 expression and ascites cytology. Survival was analyzed using the Kaplan–Meier survival analysis with the log-rank test by GraphPad Prism 5 software. A P value less than 0.05 was considered to be significant.

Results

Positive cytology of peritoneal wash or ascites is related to poor overall and progression-free survival in ovarian cancer patients

Survivals of 65 patients with ovarian cancer (KOV-IH-65) were studied. A cytologic examination at the time of operation revealed viable malignant cells in the ascites of 42 patients ("cytology-positive" cases) in this group. Positive cytology was related to poor overall survival ($P < 0.001$; Supplementary Fig. S1A) and poor progression-free survival ($P < 0.001$; Supplementary Fig. S1B) indicating that positive cytology in ascites was a significant poor prognostic factor in ovarian cancer as previously reported (1, 4).

Genes in Gene Ontology term related to immunity are enriched in cytology-positive cases

Microarray analysis of ovarian cancer tissue from 64 patients (KOV-MA-64) was conducted. Thirty patients were cytology positive in this group. Among 1,692 probes that were highly expressed in ascites-cytology–positive cases,

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genes belonging to Gene Ontology terms related to immunity, such as "regulation of immune system process," "positive regulation of immune effector process," or "regulation of IFN-γ production" were enriched. Significantly enriched Gene Ontology terms in cytology-positive cases are listed in Supplementary Table S2. PD-L1 (CD274) was included in Gene Ontology term "regulation of immune system process."

Genes upregulated by IFN-γ, including PD-L1, are enriched in cytology-positive cases

GSEA revealed that the genes upregulated in response to IFN-γ were significantly enriched in cytology-positive cases in KOV-MA-64 (Fig. 1A). FDR q value was 0.242. Genes upregulated in response to IFN-γ are shown in heatmap in Supplementary Fig. S2. Again, PD-L1 (CD274) was included in the enriched genes. These data indicate that ascites-cytology–positive cases in ovarian cancer are distinctly characterized by regulation of immune response, especially by IFN-γ-induced genes, including PD-L1.

PD-L1 protein expression in human ovarian cancer is related to positive peritoneal cytology and poor prognosis

To determine if PD-L1 protein expression also correlates to the positive peritoneal cytology, immunohistochemistry for PD-L1 in the sampled tissue was conducted (Fig. 1B). Forty-four cases were positive for PD-L1. Positive cytology cases showed tendency to have positive PD-L1 expression in the tumor tissue ($P = 0.048$, χ^2 test; $P = 0.058$, Fisher exact test; Fig. 1C).

Overall survival of PD-L1–positive patients in KOV-IH-65 was significantly shorter ($P = 0.023$) as compared with PD-L1–negative patients (Fig. 1D).

Human and mouse ovarian cancer cell lines express various levels of PD-L1

We examined the PD-L1 expression on several human and mouse ovarian cancer cell lines by flow cytometry. Two of 6 tested human cell lines expressed high levels of PD-L1, whereas 4 cell lines expressed very low levels of or no PD-L1 (Fig. 2A). The mouse ovarian cancer cell line ID8 did not express PD-L1, whereas HM-1 expressed very low level of PD-L1 (Fig. 2B).

Next, we assessed whether IFN-γ alters PD-L1 expression on these cell lines because IFN-γ is reported to induce PD-L1 expression (31, 32). Human recombinant IFN-γ (20 ng/mL) for human cells or mouse recombinant IFN-γ (20 ng/mL) for mouse cells was added to the culture medium. IFN-γ induced PD-L1 expression in 3 human cell lines and in ID8 and HM-1, whereas OV90 did not express PD-L1 even after

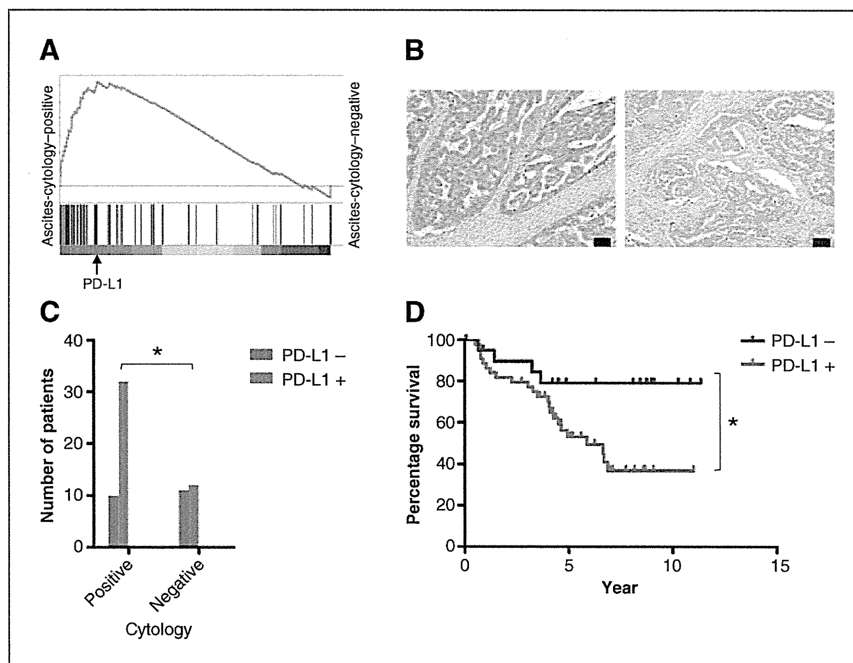

Figure 1. PD-L1 expression on human ovarian cancer cells is related to tumor survival in ascites. A, enrichment of the gene set described for response to IFN-γ in the ascites-cytology–positive cases, relative to the ascites-cytology–negative cases. Black vertical bars represent genes in this gene set. The position of the gene PD-L1 is shown by an arrow. Position to the left indicates enrichment in ascites-cytology–positive cases; a position to the right indicates enrichment in ascites-cytology–negative cases. B, PD-L1 expression in human ovarian cancer tissue. Representative samples with high expression (left) and low expression (right; magnification ×200). Bars, 50 μm. C, the result of immunohistochemistry of PD-L1 in KOV-IH-65. Positive cytology cases tend to have positive PD-L1 expression. *, $P = 0.048$, χ^2 test; $P = 0.058$, Fisher exact test. D, overall survival of KOV-IH-65. PD-L1 immunohistochemistry positive (red line) and negative (blue line). *, $P = 0.023$.

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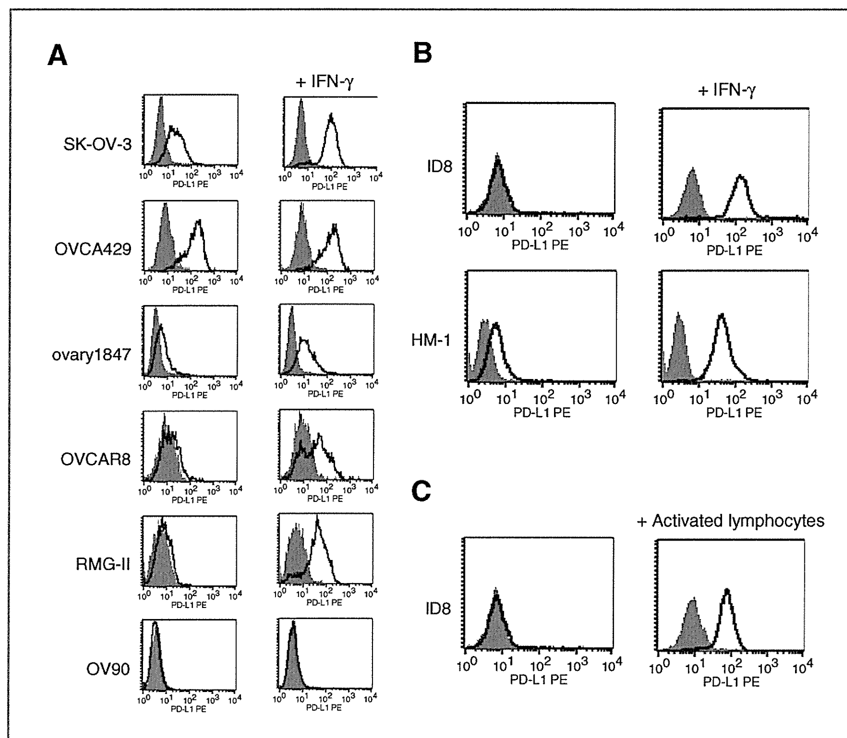

Figure 2. Human and mouse ovarian cancer cell lines express various levels of PD-L1. A, PD-L1 expression in 6 human ovarian cancer cell lines with (right) or without (left) IFN-γ exposure. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody. B, PD-L1 expression in 2 mouse ovarian cancer cell lines with or without IFN-γ exposure. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody. C, PD-L1 expression in ID8 cells coincubated with or without activated lymphocytes for 24 hours. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody.

IFN-γ exposure, indicating that this cell line has some functional loss in IFN-γ pathway (Fig. 2A and B).

Coculture with activated lymphocytes induces PD-L1 expression in mouse ovarian cancer cell lines

To determine whether activated lymphocytes, which are a possible source of IFN-γ *in vivo*, induce PD-L1 on ovarian cancer cells, we cocultured activated lymphocytes with ID8 cells. Lymphocytes from B6 mouse spleen were stimulated with 1 μg/mL of anti-mouse CD3 antibody (BioLegend) and 2 μg/mL of anti-mouse CD28 antibody (BioLegend) for 4 days before the experiment. After 24 hours of coculture, the ID8 cells were analyzed for PD-L1 expression by flow cytometry. PD-L1 expression was markedly increased after coculture with activated lymphocytes (Fig. 2C). Similarly, PD-L1 on HM-1 was also induced by coculture with syngeneic activated lymphocytes (data not shown). Thus, coculture with activated T lymphocytes induces PD-L1 in mouse ovarian cancer cells.

Ovarian cancer cells in mouse ascites express PD-L1 by encountering lymphocytes

As mouse models of ovarian cancer dissemination, ID8 and HM-1 formed cancerous ascites and massive peritoneal dissemination after intraperitoneal injection into syngeneic mice. ID8-GFP cells and HM-1-GFP cells in the ascites expressed PD-L1 (Fig. 3A), and as high as 19% of the $CD8^+$ T lymphocytes in the ascites was positive for intracellular IFN-γ (Fig. 3B). In contrast, IFN-γ concentration in ascites supernatant was very low, whereas IL-6, -10, and TNF-α were detected in higher concentrations (Fig. 3C). We tested IL-2, -6, TGF-β, TNF-α, and IL-10 to determine whether cytokines other than IFN-γ affect PD-L1 expression in the ascites, but none of the tested cytokines induced PD-L1 on HM-1 cells (Fig. 3D). As expected, adding the ascites supernatant to the culture medium did not affect PD-L1 expression on ID8 or HM-1 cells (Fig. 4A). Floating cultures in a nonadherent dish, a hypoxic culture in 1% oxygen, or both, which is a mimic of ascites condition, did not alter PD-L1 expression in HM-1 cells (Fig. 4B). However, coculture with mice ascites cells enhanced PD-L1 expression in HM-1 cells, and coculture with $CD8^+$ cells isolated from mouse ascites induced even higher levels of PD-L1 in HM-1 cells (Fig. 4C).

Administration of HM-1-GFP to a SCID mouse also forms cancerous peritonitis. However, HM-1-GFP cells in SCID mouse ascites did not express PD-L1 (Fig. 4D). These data suggest that the tumor cells express PD-L1 in ascites as a consequence of their encounter with activated lymphocytes.

Generation of PD-L1–overexpressing and PD-L1–depleted cell lines

To examine the effects of PD-L1 expression on tumor cells, we established PD-L1–overexpressing cell lines (denoted ID8-pdl1 and HM1-pdl1) and PD-L1–depleted cell lines (denoted ID8-Mirpdl1 and HM1-Mirpdl1) from the ID8 and HM-1. PD-L1 expression is shown in Supplementary Fig. S1C. To confirm that PD-L1 depletion was successfully

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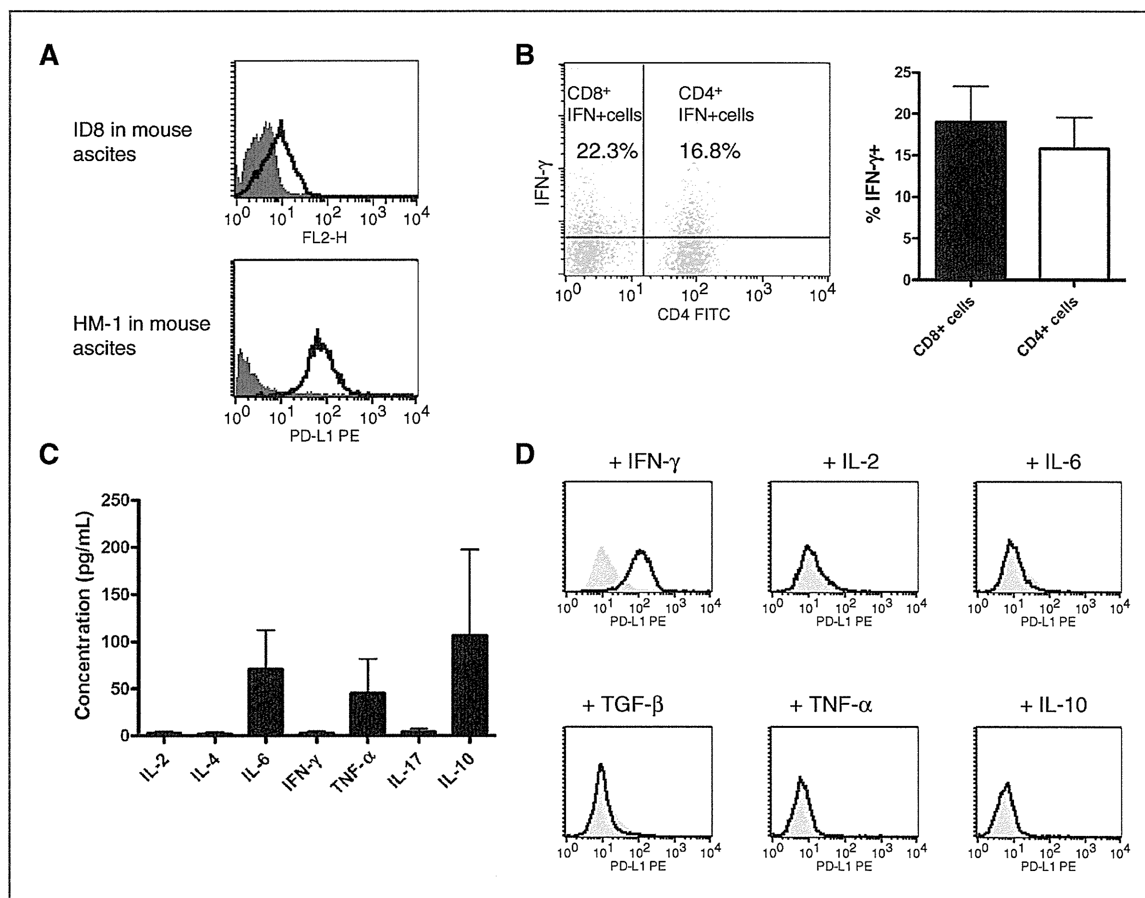

Figure 3. Ovarian cancer cells in mouse ascites express PD-L1. A, ovarian cancer cells in the ascites of the mouse ovarian cancer models express PD-L1. Flow cytometry histograms of ascites cells from a mouse inoculated with ID8-GFP (top) and HM-1-GFP (bottom) are shown. GFP-positive and 7-amino-actinomycin D–negative cells are gated as tumor cells. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody. Representative of 3 experiments with similar results. B, lymphocytes in the ascites of mouse ovarian cancer model are positive for intracellular IFN-γ. A representative result of 3 experiments (left) and percentage of intracellular IFN-γ–positive cells in mouse ascites T lymphocytes (right). Mean ± SD ($n = 3$). CD3-positive cells are gated. C, cytokine concentration in ID8-bearing mouse ascites supernatant. Mean ± SD ($n = 3$). D, PD-L1 expression after exposure to various cytokines. None of the tested cytokines other than IFN-γ induced PD-L1 on HM-1. Shaded histogram, PD-L1 expression without cytokine; open histogram, PD-L1 expression with cytokine added to the medium 24 hours before the assessment.

achieved, PD-L1–depleted cell line or control cell line was coincubated with ascites cells or ascites $CD8^+$ cells, and PD-L1 expression in the depleted cell line was lower than in the control cell lines (Supplementary Fig. S1D).

In vitro cell proliferation is not affected by PD-L1 expression

A cell proliferation assay revealed that the proliferation curves of the PD-L1–manipulated cell lines were similar to those of the control cell lines (Fig. 5A), indicating that PD-L1 expression does not affect cell proliferation *in vitro*.

PD-L1 protects ovarian cancer cells from antigen-specific cytolysis by CTLs

We next conducted a cytotoxicity assay to examine antigen-specific cytolysis by $CD8^+$ CTLs. The cytotoxicity curves were significantly different between the cell lines. High levels of target cell lysis were observed in ID8-Mirpdl1 cells, and low levels of target cell lysis were observed in ID8-pdl1 cells (Fig. 5B), indicating that antigen-specific cytolysis by CTLs is inhibited by PD-L1 and can be promoted by PD-L1 depletion.

CTL function is inhibited by tumor-associated PD-L1

Alterations in CTLs following their encounter with tumor-associated PD-L1 were assessed. CTLs lyse target cells by secreting perforin and granzymes, and CD107a is a surface marker for the degranulation of activated CTLs. CD107a expression in the CTLs cocultured with ID8-pdl1 was weaker than control, indicating that T-cell degranulation following antigen stimulation has been inhibited by tumor-associated PD-L1 (Fig. 5C). Under microscopic

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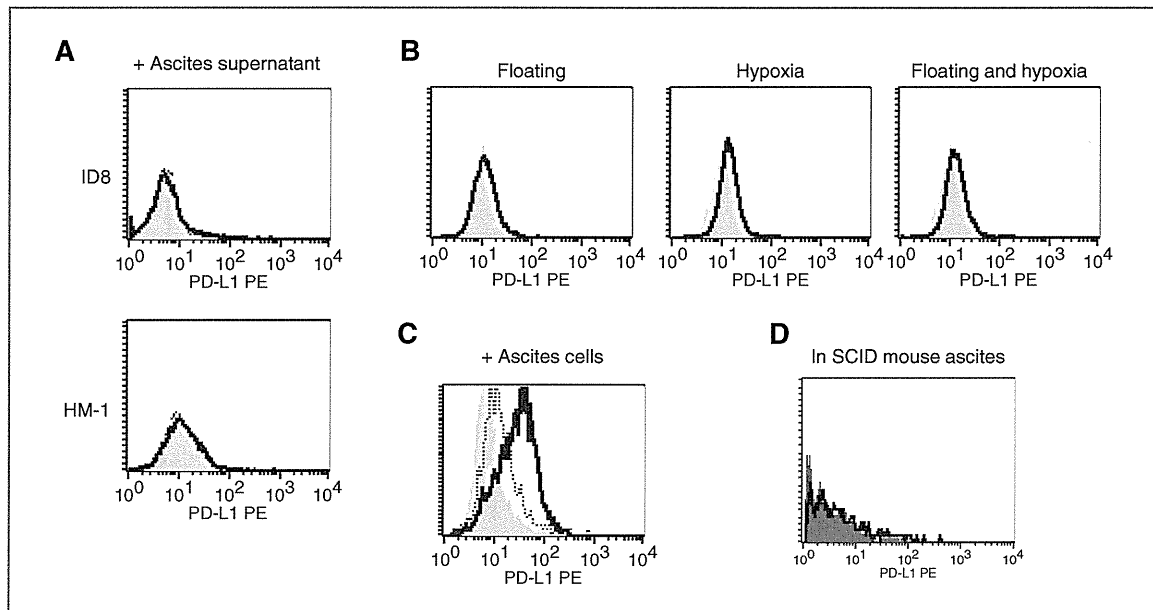

Figure 4. Lymphocytes in ascites induce PD-L1 on mouse ovarian cancer cells. A, ascites supernatant did not induce PD-L1 in ID8 or HM-1 cells. Shaded histogram, isotype control; open histogram, anti-PD-L1 antibody. Representative of 3 repeated independent experiments with similar results. B, PD-L1 expression under various culture conditions. Floating culture in nonadherent dish, culture under hypoxic condition (1% O_2), or both did not affect PD-L1 expression. Shaded histogram, PD-L1 expression in normal culture condition; open histogram, PD-L1 expression under floating, hyoxic, or both floating and hypoxic conditions. Representative of 3 repeated independent experiments with similar results. C, $CD8^+$ T cells from mouse ascites induce PD-L1 expression on HM-1. Shaded histogram, cultured without any ascites cells; dotted line histogram, ascites cells added to the culture; solid line histogram, ascites $CD8^+$ cells added to the culture. Representative of 3 repeated independent experiments with similar results. D, mouse ovarian cancer cells in SCID mouse ascites do not express PD-L1. Representative of 3 mice with similar results.

observation while coculturing with these target cells, CTLs gathering to the tumor cells were markedly inhibited in ID8-pdl1 (Fig. 5D and Supplementary Videos S1 and S2). These results indicate that PD-L1 on tumor cells inhibit CTL function.

Gene expression profile of mouse CTLs affected by PD-L1 shows correlations to PD-1 signal genes in human

PD-L1 is reported to transmit an inhibitory signal through its receptor, PD-1, in lymphocytes. To examine the alteration in gene expression profiles in mouse CTLs associated with PD-L1, microarray analysis for CTLs coincubated with ID8-pdl1 or ID8-Mirpdl1 was conducted, and the gene expression profile was compared by GSEA with a publicly accessible gene set of human functionally impaired $CD8^+$ T cells by positive PD-1 signal (30). Up- and down-regulated genes in mouse CTLs are shown in Supplementary Table S3. Interestingly, the genes upregulated in PD-L1–affected mouse CTLs were significantly enriched in upregulated genes in PD-1 downstream genes in human CTLs. Furthermore, the genes downregulated in PD-L1–affected mouse CTLs were also significantly downregulated in PD-1 signal-transmitted human CTLs (Supplementary Table S4). This result is consistent with the fact that PD-L1 on tumor cells transfers inhibitory signal through PD-1 on CTLs and also validate the similar mechanism of PD-L1/PD-1 effect in human and mouse CTLs.

PD-L1 promotes tumor progression in mouse ovarian cancer dissemination models

HM-1-pdl1, HM-1-Mirpdl1, or HM1-control cells were injected intraperitoneally to syngeneic mice. After 7 days, the body weight of the mice, a reliable marker of tumor growth, in all 3 groups increased (Fig. 6A). However, in the HM1-Mirpdl1 group, the body weight decreased after 10 days (Fig. 6A, right). Therefore, PD-L1 depletion decreased the tumor that once grew in the peritoneal cavity.

The survival of the mice is shown in Fig. 6B–D. The HM-1-pdl1 group lived shorter ($P = 0.039$) than the control group, and the HM-1-Mirpdl1 group lived longer ($P = 0.0029$; Fig. 6B). In ID8-injected mice, the survival of the ID8-pdl1 group and the ID8-control group were similar (Fig. 6C), indicating that differences in PD-L1 expression upon injection is eventually abrogated in slow-progressing tumors because PD-L1 is induced in the peritoneal cavity. However, the mice in the ID8-Mirpdl1 group had significantly longer survival times than the control group ($P < 0.001$; Fig. 6C). PD-L1 expression in tumor cells did not affect the survival of SCID mice following intraperitoneal injection (Fig. 6D).

Discussion

Although various molecules expressed by cancer cells have been implicated in the process of peritoneal dissemination, the influence of immunologic factors is poorly understood. In this study, we first focused on the state of

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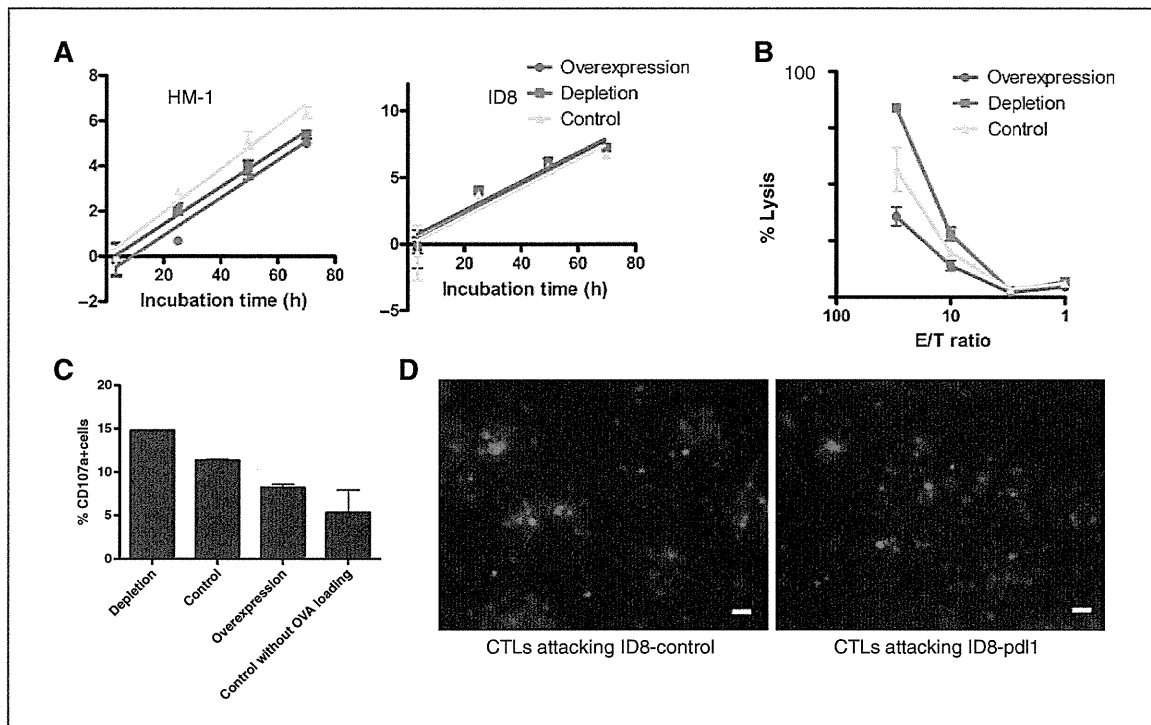

Figure 5. PD-L1 protects tumor cells from CTLs. A, cell proliferation assay of the PD-L1–manipulated HM-1 cell lines (left) and ID8 cell lines (right). *Y*-axis, relative number of cells in $\log_2$ scale. *X*-axis, incubation time (h). Mean ± SD ($n = 6$) from 1 representative experiment of 2 repeated experiments with similar results. Relative number of cells is calculated in the following formula: (Relative number of cells) = (number of cells estimated by water soluble tetrazolium–8 assay)/(seeded cells). B, cytotoxicity assay of the PD-L1–manipulated ID8 cell lines. Mean ± SD ($n = 4$) from 1 representative experiment of 3 repeated experiments with similar results. C, CD107a$^+$ CTLs following coincubation with OVA-loaded ID8-Mirpdl1, OVA-loaded ID8-control, OVA-loaded ID8-pdl1, or ID8-control without OVA loading. Mean ± SD ($n = 3$) from 1 of 3 repeated experiments with similar results. D, microscopic image of activated GFP+ CTLs, after 136 minutes of coincubation with ID8-control (left) or ID8-pdl1 (right). Bars, 50 μm. Time-lapse video available in Supplementary Videos S1 and S2. One of 3 repeated experiments with similar results.

"positive peritoneal cytology," which represents the status that the tumor cells are surviving in peritoneal cavity without being destroyed by host immunity. We confirmed that positive cytology adversely affects the overall and progression-free survival of the patients. Then, we analyzed PD-L1 expression in the primary tumor, both in mRNA and protein levels, and found for the first time that it significantly correlates to positive peritoneal cytology. Furthermore, in microarray analyses, gene profile associated with positive peritoneal cytology was significantly enriched of immune-related genes, including PD-L1, assessed by a Gene Ontology analysis. An IFN-γ–induced gene signature, which also includes PD-L1, was also significantly associated with positive peritoneal cytology by GSEA. Together, these data imply that peritoneal spread of ovarian cancer accompanies with local immune modification, and that PD-L1 functions as a key molecule in this process. These data prompted us to further investigate the function of PD-L1 in ovarian cancer cells, especially as related to the peritoneal dissemination.

The mechanism by which PD-L1 expression is regulated is quite ambiguous, especially in cancer cells. In an early report, PD-L1 was reported to be expressed only in immune cells under natural circumstances and to be highly expressed in some tumor cells (31). Subsequent reports have shown that PD-L1 is expressed constitutively in some normal tissues including eyes and placenta (33, 34), and that PD-L1 can be induced in cancer cells and noncancer cells by IFN-γ (35, 36). However, the precise mechanism of PD-L1 induction, especially *in vivo*, is still unclear. Therefore, we initially examined PD-L1 expression under natural culture conditions as well as upon various cytokine stimulations, including IFN-γ, in 6 human and 2 mouse ovarian cancer cell lines. The results suggest that there are 3 types of cells with regards to PD-L1 expression: type A cells (e.g., SK-OV-3) always express PD-L1; type C cells (e.g., OV90) never express PD-L1; and type B cells (e.g., OVARY1847) do not express PD-L1 at baseline but express PD-L1 when exposed to IFN-γ. Type B was most frequent in the tested human ovarian cancer cell lines. It is assumed that PD-L1 expression is not constitutive in these cells but is induced by the influence of other factors. In a mouse experiment, we used 2 type B mouse ovarian cancer cells, ID8 and HM-1. Both cell lines expressed PD-L1 when administered into the

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