rates of interstitial lung disease associated with gefitinib and leflunomide are 2 and 1.1% in Japan and 0.3 and 0.02% in the United States, respectively. These data indicate that chemotherapeutic-drug-induced pulmonary toxicity is more frequent in Japan than in other nations [9, 10]. Fatal pneumonitis induced by gefitinib or leflunomide is less frequent in other Asian countries than in Japan. It may be that such drugs including PLD cause fatal pneumonitis predominantly in Japanese. The differences of genetic background or lifestyle between Japanese and non-Japanese might be involved in this event.

Drug-induced interstitial pneumonitis should be taken into consideration in the differential diagnosis of otherwise unexplained ground-glass lung lesions. Pulmonary toxicity induced by PLD is rare, but awareness of this toxicity is important, since it could be lethal. Additional investigation is required to elucidate how PLD induces interstitial pneumonitis or whether PLD-induced interstitial pneumonitis is more frequent in Japanese.

Conflict of interest No author has any conflict of interest.

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LETTER TO THE EDITOR

Second-line chemotherapy with docetaxel and carboplatin in paclitaxel and platinum-pretreated ovarian, fallopian tube, and peritoneal cancer

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Abstract We retrospectively evaluated the efficacy and toxicity of docetaxel and carboplatin in patients with platinum and paclitaxel-pretreated recurrent ovarian, fallopian tube, and peritoneal cancer. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four patients had measurable disease. The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, 34 weeks, and the median overall survival time was 94, 224, 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes (8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months.

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The standard regimen as second-line chemotherapy in recurrent ovarian cancer has not been established, especially in the patients with a short progression-free interval from the previous treatment. Docetaxel is an active drug as second-line chemotherapy for recurrent ovarian cancer as well as pegylated liposomal doxorubicin, irinotecan, topotecan, gemcitabine, and etoposide [1].

The purpose of this study was to evaluate activity and toxicity of the combination of docetaxel and carboplatin retrospectively in patients with paclitaxel and platinum resistant (progression-free interval less than 6 months) and partially resistant (progression-free interval of 6–12 months) ovarian, fallopian tube, and peritoneal cancers. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four (81%) patients had measurable disease. Twenty-six (62%) patients had experienced progression of disease within less than 6 months of their last treatment, whereas 16 patients (38%) within 6–12 months. The median number of courses of treatment per patient was 4.5 (range: 1–8 courses). The median follow-up period was 107 weeks (range: 9–373 weeks). The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, and 34 weeks, and the median overall survival time was 94, 224, and 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes

(8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. On the other hand, grade 2/3 neuropathy was observed only in two (4.8%) patients.

Several chemotherapeutic agents such as pegylated liposomal doxorubicin, topotecan, irinotecan, gemcitabine, and etoposide have been used in the treatment of platinum-resistant disease with response rates in the range 10–15% [2–5]. The results from our study about overall response rate are in line with other chemotherapeutic agents. Notably, our data about median time to tumor progression and overall survival are longer than the previously reported data of other regimens.

The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months. However, chemotherapy with docetaxel and carboplatin may improve time to tumor progression and overall survival time in these cases; this regimen can be an alternative in patients whose hematological toxicity is relatively weak at their previous treatment.

Conflict of interest None.

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Do Neutralizing Antibody Responses Generated by Human Papillomavirus Infections Favor a Better Outcome of Low-Grade Cervical Lesions?

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To determine the role of neutralizing antibody generated by human papillomavirus (HPV) infections, baseline levels of serum neutralizing antibodies directed against HPV 16 and cervical HPV DNA were determined in 242 unvaccinated women with low-grade cervical abnormalities, who were then monitored by cytology and colposcopy every 4 months. In women infected with HPV 16 (n = 42), abnormal cytology persisted longer in those positive for HPV 16-specific neutralizing antibodies at baseline (median time to cytological regression: 23.8 vs. 7.2 months). Progression to cervical precancer (cervical intraepithelial neoplasia grade 3) within 5 years occurred only among women carrying HPV 16-specific neutralizing antibodies ($P = 0.03$, log-rank test). In women infected with types other than HPV 16 (n = 200), detection of HPV 16-specific neutralizing antibodies was not correlated with disease outcome. In conclusion, development of specific neutralizing antibodies following natural HPV 16 infection did not favor a better outcome of low-grade cervical lesions induced by HPV 16 or by other types; rather, detection of neutralizing antibodies generated by current infection may reflect viral persistence and thus help identify those who are at high risk of disease progression. ***J. Med. Virol. 84:1128–1134, 2012.***

KEY WORDS: human papillomavirus; neutralizing antibody; low-grade squamous intraepithelial lesion; progression; cervical intraepithelial neoplasia

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INTRODUCTION

Human papillomavirus type 16 (HPV 16) is the most common genotype detected in cervical cancer worldwide [Muñoz et al., 2003]. HPV 16 virus-like particles (VLPs) are obtained through the self-assembly of the major capsid protein L1 expressed in insect or yeast cells. HPV 16 VLP-based ELISA has been used to detect HPV 16 capsid antibody responses following natural infections [Frazer, 2009]. More than half of women infected by HPV 16 produce serum IgG antibodies [Carter et al., 2000]. HPV 16 VLP IgG antibodies are more commonly detected in women who have been persistently positive for HPV 16 DNA at different time points [de Gruijl et al., 1997; Sasagawa et al., 1998]. In women not infected currently with HPV 16, the presence of ELISA antibody responses to HPV 16 VLPs correlated with a significantly reduced risk of subsequent new HPV 16 infection, although the risk was reduced by only 50% compared to that of seronegative women [Ho et al., 2002; Safaeian et al., 2010].

In the case of many viruses, the presence of serum neutralizing antibodies is a correlate of immune protection. Recently, production of HPV 16 pseudovirions encapsidating a secreted alkaline phosphate (SEAP) reporter gene has allowed efficient measurement of specific HPV 16 neutralizing antibodies in women infected with HPV 16 or vaccinated with HPV 16 VLPs. Although serum antibody titer measured by VLP-based ELISA correlated with the neutralizing antibody titer, the neutralization assay was found to be more sensitive and type-specific than the VLP-based ELISA detecting total antibodies to HPV 16 capsids [Pastrana et al., 2004]. Immunization with VLP-based vaccines elicits neutralizing antibodies and protects against new infections in animals and humans [Stanley, 2010]. However, there have been few studies assessing the role of neutralizing antibodies in women infected currently with HPV or in those with cervical diseases. The neutralization activity against HPV may prevent persistence of infection by inhibiting viral spread between cervical epithelial cells or act as markers of host immunity responses to control cervical HPV infection.

Most low-grade cervical lesions are known to regress spontaneously, whereas only a small fraction progress to precancer lesions and cervical cancer [Östör, 1993; Melnikow et al., 1998; Holowaty et al., 1999]. In the current study, baseline serum neutralizing antibodies against HPV 16 were measured to assess possible association with clinical outcomes of low-grade cervical lesions induced by HPV 16 or other HPV types.

MATERIALS AND METHODS

Study Design

Follow-up data from the Japan HPV And Cervical Cancer (JHACC) study (a prospective nonintervention cohort study conducted to assess regression and progression of low-grade cervical abnormalities [Matsumoto et al., 2010, 2011]) were used in this study. Among a total of 570 study subjects, 242 women, whose serum samples were available for the HPV 16 neutralizing assay, were enrolled in the present study. Details of the design and methods have been provided elsewhere [Matsumoto et al., 2011]. Briefly, women with low-grade squamous intraepithelial lesions which were diagnosed as cervical intraepithelial neoplasia grade 2 or less on initial biopsy were recruited from nine hospitals. All volunteer patients provided written informed consent. Cervical smears were classified according to the Bethesda System [Solomon et al., 2001]. On enrollment, two cervical punch biopsy specimens were collected and stained with hematoxylin and eosin (H&E). Histological diagnosis was based on the World Health Organization (WHO) classification system. All cytological and histological specimens were reviewed by two cytopathologists (Y. H. and Masafumi Tsuzuku) and two pathologists (R. F. and T. K.). Levels of serum neutralizing antibodies to HPV 16 and cervical HPV DNA were also determined on enrollment. Researchers conducting the assays were blinded to the corresponding clinical data collected from the patients. The patients meeting enrollment criteria were followed at 3- to 4-month intervals with cytology and colposcopic examination. Cervical HPV DNA was determined at 24 months after enrollment. To avoid interference with the natural course of the disease, a cervical biopsy was performed only when the follow-up findings suggested progression to cervical intraepithelial neoplasia grade 3 or worse. The two cytopathologists and two pathologists reviewed all the cytological and histological specimens collected for diagnosis of disease progression. Progression was defined histologically as the presence of cervical intraepithelial neoplasia grade 3 lesions. In this analysis, regression was defined as normal colposcopy and at least two consecutive negative cervical smears. Lesions which did not regress or progress during the follow-up period were defined as persistent lesions.

The study protocol was approved by the ethics and research review boards of the participating institutions.

HPV Genotyping

HPV DNA in cervical samples was determined by the polymerase chain reaction (PCR), as described previously [Yoshikawa et al., 1991]. Briefly, exfoliated ecto- and endocervical cells were placed in a tube containing 1 ml phosphate buffered saline and stored at −30°C until DNA extraction. Total cellular DNA was extracted by a standard sodium dodecyl sulfate (SDS)–proteinase K procedure. HPV DNA was amplified by PCR using consensus-primers (L1C1/L1C2 + L1C2M) for the HPV L1 region. A reaction mixture without template DNA was included in every

set of PCR runs as a negative control. In addition, primers for a fragment of the β-actin gene were used as an internal control to assess the quality and quantity of template DNA in each PCR specimen. HPV types were identified by restriction fragment length polymorphism (RFLP), which has been shown to distinguish at least 26 types of genital HPVs [Nagano et al., 1996].

To minimize misclassification errors of HPV 16 DNA, HPV 16 infection was confirmed by PCR-amplified DNA sequencing using HPV 16 E6-specific synthetic primers (5-GACATTTTATGCACCAAAAG-3 and 5-GTATCT CCATGCATGATTAC-3, spanning nt 75–575) [Matsumoto et al., 2000].

Preparation of Pseudovirions

Dr. J. T. Schiller kindly donated three plasmids for this study: pYSEAP, expressing secreted alkaline phosphatase (SEAP); p16L1h, expressing HPV 16 L1; and p16L2h, expressing HPV 16 L2. 293FT cells (Invitrogen, Carlsbad, CA) cultured in two 10-cm culture dishes (6×10^6 cells/dish) for 16 hr were transfected with a mixture of a p16L1h plasmid (13.5 μg), a p16L2h plasmid (3 μg), and pYSEAP plasmid (13.5 μg) using Fugene HD (Rosch Diagnostics, Mannheim, Germany). After 60 h, pseudovirions (PVs) were purified from the cells, as described previously [Kondo et al., 2007], and their infectivity was estimated from the SEAP activity in culture media of infected cells using a colorimetric assay.

Neutralization Assay

The neutralization assay was performed as described previously [Ochi et al., 2008]. Briefly, 50 μl of serum was diluted with 50 μl of neutralization medium (DMEM [without phenol red], 10% FBS, 1% nonessential amino acids, 1% GlutaMax-I) containing an aliquot of the PV stock (400 pg of HPV 16 L1) giving an optical density of approximately 1.0 in the SEAP activity assay conditions and then incubated for 1 h at 4°C. The amount of PV used was in the linear range of the dose–response curve. The mixture was then inoculated onto 293FT cells that had been cultured in 96-well plates (1×10^4 cells/well) for 16 h. Culture media were harvested after 4.5 days at 37°C, and SEAP activity was determined. The neutralization titer is presented as the reciprocal of the maximum dilution of serum that reduced SEAP activity to half the level of that in the control samples.

Statistical Analysis

All time-to-event analyses were based on the actual date of visits. For regression or progression, time to event was measured from the date of the index visit (i.e., the first instance of an abnormal cytology result) to the date of the visit at which cytological transition to normal or to cervical intraepithelial neoplasia grade 3 was first detected. Women whose lesions persisted or who dropped out of the study were censored at their last recorded return visit dates. Patients who had only one negative colposcopy/cytology result before loss to follow-up were censored at the last date of positive Pap tests. Subjects who were biopsied were censored at the time of their biopsy, regardless of the biopsy results, to reduce the potential for interference by the biopsy procedure on estimates of time of regression. Cumulative probability of disease regression or progression was estimated using the Kaplan–Meier method and compared with a log-rank test, and the Cox regression model was used for statistical adjustments. Patient age (18–29, 30–39, or 40–54 years of age) and initial biopsy results (cervical intraepithelial neoplasia grade 1 or 2) were included in the multivariate model for adjustments. The χ^2 or Fisher's exact test was used to determine whither presence of HPV 16-specific neutralizing antibodies is significantly associated with risk of viral persistence. All analyses were carried out using the JMP 7.0J statistics packages (SAS Institute, Cary, NC). Two-sided P-values were calculated throughout and differences were considered significant for $P < 0.05$.

RESULTS

A total of 242 women with low-grade squamous intraepithelial lesions (206 with cervical intraepithelial neoplasia grade 1 and 36 with grade 2) were enrolled in this study. Mean age was 35.7 years (range 19–52 years). The total number of clinical visits was 3850 and the mean follow-up time was 46.2 months (median 43.2; range 6.8–84.9). During the follow-up period, 26 lesions progressed to cervical intraepithelial neoplasia grade 3, and 159 spontaneously regressed to normal cytology. No progression to invasive cancer was observed.

Detection of serum neutralizing antibodies against HPV 16 was strongly associated with the presence of viral DNA in the cervix (Table I), consistent with previous reports [Ochi et al., 2008]. The detection rate of HPV 16-specific neutralizing antibodies was much higher in women infected with HPV 16 than in those infected with other HPV types or those without any HPV DNA (59.5% vs. 12.5%, $P < 0.0001$ by χ^2 test). HPV 16-specific neutralizing antibodies were detected in 10.4% (19/182) of women with HPV strains other than HPV 16 and in 33.3% (6/18) of those without any HPV DNA. In the great majority of those with HPV 16 neutralizing antibodies, titers ranged between 40 and 320. High-titer (≥1,280) neutralizing sera were found in two women infected with HPV 16 and in one without any HPV DNA.

In those infected with HPV 16, a trend was observed for longer persistence of low-grade squamous intraepithelial lesions in HPV 16-specific neutralizing antibody carriers (median time to cytological regression: 23.8 months vs. 7.2 months, Fig. 1A), but the difference did not reach statistical significance ($P = 0.18$). Statistical adjustments for age and initial

TABLE I. The Association Between Cervical HPV Genotypes and Neutralizing Antibodies Against HPV 16

		Neutralizing antibodies against HPV 16							
		Neutralization		Neutralizing titer					
Cervical HPV genotype	N	Negative	Positive (%)	40	80	160	320	640	≥1,280
HPV 16 DNA positive	42	17	25 (59.5%)*	7	6	2	6	2	2
HPV 16 alone	34	13	21 (61.8%)	4	5	2	6	2	2
Multiply infected	8	4	4 (50.0%)	3	1	0	0	0	0
HPV 16 DNA-negative	200	175	25 (12.5%)*	4	8	6	2	3	1
Negative	18	12	6 (33.3%)	0	1	1	0	2	1
HPV 6	2	2	0 (0.0%)	0	0	0	0	0	0
HPV 18	9	8	1 (11.1%)	0	0	0	1	0	0
HPV 31	5	4	1 (20.0%)	0	1	0	0	0	0
HPV 33	2	1	1 (50.0%)	1	0	0	0	0	0
HPV 35	4	4	0 (0.0%)	0	0	0	0	0	0
HPV 39	4	4	0 (0.0%)	0	0	0	0	0	0
HPV 51	29	27	2 (6.9%)	1	1	0	0	0	0
HPV 52	35	31	4 (11.4%)	0	2	1	0	1	0
HPV 53	4	4	0 (0.0%)	0	0	0	0	0	0
HPV 56	23	22	1 (4.3%)	0	1	0	0	0	0
HPV 58	24	22	2 (8.3%)	1	1	0	0	0	0
HPV 59	2	1	1 (50.0%)	0	1	0	0	0	0
HPV 61	1	1	0 (0.0%)	0	0	0	0	0	0
HPV 66	6	5	1 (16.7%)	0	0	1	0	0	0
HPV 68	2	2	0 (0.0%)	0	0	0	0	0	0
Undetermined	14	12	2 (14.3%)	0	0	1	1	0	0
Multiple infection	16	13	3 (18.8%)	1	0	2	0	0	0

*The difference was statistically significant ($P < 0.0001$ by χ^2 test).

biopsy results (cervical intraepithelial neoplasia grade 1 or 2) did not change this finding (data not shown). Interestingly, progression to cervical precancer occurred only in those who had HPV 16-specific neutralizing antibodies at the baseline (Fig. 1B), and the associated risk of progression was statistically significant ($P = 0.03$ by log-rank test). Among women with cervical intraepithelial neoplasia grade 2 lesions, all (3/3, 100%) who had HPV 16-specific neutralizing antibodies at the baseline were diagnosed with grade 3 lesions within 5 years, while no such progression was observed in those who did not have HPV 16 neutralizing antibodies (0/5, 0%) ($P = 0.01$ by log-rank test). Among women with cervical intraepithelial neoplasia grade 1, 13.6% (3/22) positive for HPV 16 neutralizing antibodies were diagnosed with grade 3 disease within 5 years, while no such progression was found in those without HPV 16 neutralizing antibodies (0/12, 0%) ($P = 0.20$ by log-rank test). Adjusted P-values could not be calculated in the Cox proportional hazard model because no event occurred among women without serum HPV 16 neutralizing antibodies.

Neutralizing antibody responses resulting from previous HPV 16 clearance did not favor better outcomes of cytological abnormalities induced by other HPV types. The probability of disease regression within 2 years was not significantly different between women with or without serum HPV 16-specific neutralizing antibodies (44.0% vs. 57.0%, $P = 0.35$ by log-rank test). There was also no significant difference between these two groups in probability of progression to cervical intraepithelial neoplasia grade 3 within 5 years (8.3% vs. 12.0%, $P = 0.44$ by log-rank test). Analyses confined to the various cervical HPV 16-related types studied (HPV 31, HPV 33, HPV 35, HPV 52, and HPV 58) or statistical adjustment for age and initial biopsy results did not change these findings (data not shown).

HPV DNA data at baseline and 24 months were analyzed to determine whether detection of HPV 16-specific neutralizing antibodies was associated with persistent HPV infections. Results at 24 months were available for 149 women. Persistent infection was defined as continued detection at 24 months of HPV genotypes present at baseline. Among women infected with HPV 16 (n = 26), HPV 16 persistence was more common in those positive for serum HPV 16 neutralizing antibodies than in those with none (61.5% vs. 15.4%, Fisher's exact test: $P = 0.04$). Women who had serum HPV 16 neutralizing antibodies were found to be at a much higher risk of persistent infection compared with those who had none (odds ratio 8.06, 95% confidence interval 1.51–51.3). Among women infected with other HPV types (n = 123), HPV 16 neutralizing antibodies were not associated with persistent infections by baseline HPV genotypes (χ^2 test, $P = 0.61$).

DISCUSSION

In women with low-grade cervical lesions induced by HPV 16, progression to cervical precancer (cervical intraepithelial neoplasia grade 3) occurred only

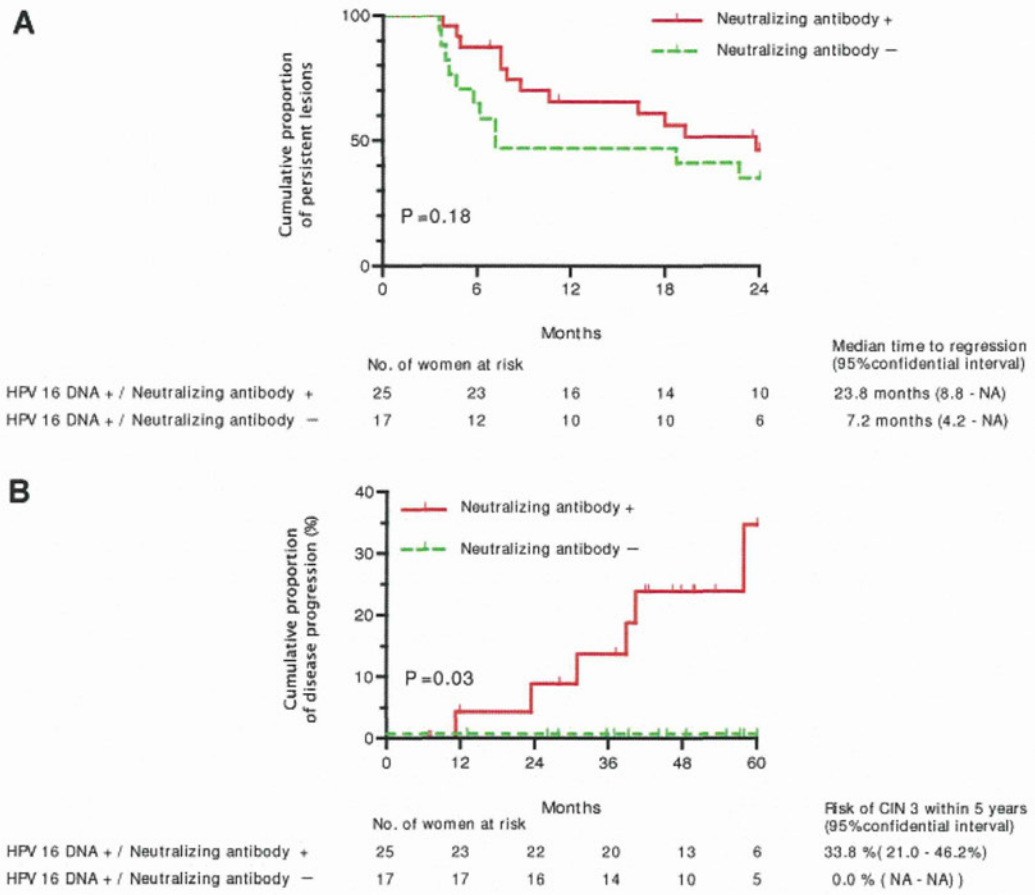

Fig. 1. Neutralizing Antibody against HPV 16 and Natural History of Low-grade Squamous Intraepithelial Lesion induced by HPV 16. Kaplan–Meier plots were used to estimate cumulative probabilities of regression (**A**) and progression (**B**) of low-grade squamous intraepithelial lesion induced by HPV 16 in relation to HPV 16 neutralizing antibody status. *P*-values calculated by log-rank test. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jmv]

among women carrying HPV 16-specific neutralizing antibodies. This finding is consistent with two previous reports of ELISA-based studies: HPV 16 VLP-specific antibodies were detected in all women whose disease eventually progressed to cervical precancer during follow-up, although results of statistical tests were not shown [de Gruijl et al., 1997; Sasagawa et al., 1998]. The use in this study of a neutralization assay which is more sensitive and type-specific than the VLP-based ELISA [Pastrana et al., 2004] demonstrated that the presence of neutralizing antibodies to HPV 16 confers a significantly higher risk of progression to cervical precancer among women with low-grade cervical abnormalities induced by HPV 16 ($P = 0.03$). The reason for this association may be that generation of antibodies to HPV 16 capsids reflects viral persistence. In the ELISA-based studies, HPV 16 capsid antibodies were more frequently detected in women who were persistently HPV 16 DNA-positive [de Gruijl et al., 1997; Sasagawa et al., 1998]. In the present study, low-grade cervical lesions were more likely to persist longer among women with HPV 16-specific neutralizing antibodies. These observations suggest that testing for VLP-specific or neutralizing antibodies to cervical HPV infection may identify women who are at high risk of viral persistence and disease progression. Although it was reported previously that type-specific HPV testing for women with low-grade cervical lesions is useful for identifying populations at increased risk of disease progression [Matsumoto et al., 2011], combined HPV typing and serological assays may aid more accurate stratification according to risk of disease persistence and progression.

An earlier study demonstrated that detection of HPV 16 neutralizing antibodies in sera was significantly associated with spontaneous regression of cervical intraepithelial neoplasia grade 1 lesions induced by HPV 16 [Kawana et al., 2002]. This result is contrary to the findings of the present study. The discrepancy may be explained in part by differences in the neutralization assays employed. The SEAP-mediated neutralization assay has been demonstrated to be more sensitive and type-specific than the assay used previously [Pastrana et al., 2004]. Indeed, the detection rate of HPV 16 neutralizing antibodies among

women with cervical intraepithelial neoplasia grade 1 induced by HPV 16 was much higher in the present than the previous study [Kawana et al., 2002] (64.7% vs. 21.6%). The present results are consistent with a recent report indicating that serum HPV 16 neutralizing antibodies were detected in 77.7% of women with cervical precursor lesions induced by HPV 16 [Mbulawa et al., 2008]. Furthermore, the present results were obtained from more extensive long-term follow-up data. In the present study, all cytological and histological data were reviewed by two cytopathologists and two pathologists. The median follow-up period was longer in this study compared to that of the previous report (46 vs. 24 months), which allowed for investigation of the association between HPV 16-specific neutralizing antibody and disease progression. The present findings are also supported by several previous ELISA-based studies of women with low-grade cervical lesions [de Gruijl et al., 1997; Sasagawa et al., 1998; Matsumoto et al., 2006] in which VLP-specific IgG antibodies were not associated with spontaneous regression of low-grade cervical abnormalities.

The presence of serum HPV 16-specific neutralizing antibodies in women with low-grade cervical lesions induced by other HPV types suggests that exposure to a given type may not protect against infections by other types and subsequent development of cervical lesions. In addition, the present study also demonstrated that neutralizing antibody responses to a given type did not influence the clinical outcomes of cervical precursor lesions induced by other types. This finding suggests that host adaptive immunity arising from previous viral clearance may not favor the clearance of low-grade cervical lesions induced by other types. This may be explained by several findings that epithelial cells expressing HPV antigens may not reactivate memory CTL due to impaired antigen presentation [Matsumoto et al., 2004; Wolkers et al., 2004]. Alternatively, immune responses inducing regression of low-grade cervical lesions may be HPV type-specific, which would have important implications for the design of therapeutic vaccines against cervical intraepithelial neoplasia [Kadish et al., 1997].

In the present study, the detection rate of HPV 16 neutralizing antibodies among women without HPV DNA was relatively high (33.3%, 6/18). These women did not have HPV 16 DNA by both PCR methods. Among the women without any HPV DNA, abnormal cervical cytology persisted for more than 24 months in 2 of 6 women having HPV 16 neutralizing antibodies but in none of 12 women lacking HPV 16 neutralizing antibodies ($P = 0.03$). Since detection of serum HPV 16 neutralizing antibodies strongly correlates with the presence of HPV 16 DNA in the cervix [Mbulawa et al., 2008; Ochi et al., 2008], the finding of HPV DNA negativity among some women positive for HPV 16 neutralizing antibodies might be due to sampling errors in the collection of cervical specimens.

VLP-based vaccination eliciting high titers of neutralizing antibodies have no therapeutic effect on an existing viral infection in animals and humans [Kirnbauer et al., 1996; Hildesheim et al., 2007]. Clearance rates of HPV 16 and HPV 18 infections at 6 and 12 months were similar between vaccinated and unvaccinated women, indicating that neutralizing antibodies elicited by VLP-based vaccination does not affect either viral clearance or persistence [Hildesheim et al., 2007]. In the present study, however, the HPV 16 neutralizing antibody response induced by natural infections was significantly associated with HPV 16 persistence. These observations suggest that HPV neutralizing antibodies induced by natural infection may be immunologically quite different from those elicited by vaccination.

To date, to the best of our knowledge, no measured immune response has been shown to define immunological control of established HPV infections. Available neutralization assays are useful for estimating protective immunity in vaccinated women. However, neutralizing antibodies elicited by current viral infection or previous clearance of other HPV types did not serve as a marker of the host's ability to control the viral infection and its associated cervical disease. The available serological assays provide only a partial characterization of host immune status and VLP-specific immune responses seem unrelated to the process of viral clearance.

In summary, a serum neutralizing antibody response against HPV 16 did not favor a better outcome for low-grade cervical lesions induced by HPV 16 or by other HPV types; rather, detection of neutralizing antibodies against cervical HPV may help identify women who are at high risk of viral persistence and disease progression. Although the present data suggest the potential usefulness of combined HPV DNA genotyping and type-specific neutralization assays in the management of women with low-grade cervical lesions, further studies are warranted to validate these results.

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ORIGINAL PAPER

Aromatase inhibitor anastrozole as a second-line hormonal treatment to a recurrent low-grade endometrial stromal sarcoma: a case report

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Abstract Low-grade endometrial stromal sarcoma (ESS) is a rare neoplasm and is generally an indolent tumor with estrogen and progesterone receptors. Objective responses by hormonal treatment with progestin or aromatase inhibitor have been reported, however, long-term management of this disease could be difficult if it becomes refractory to one of these hormonal therapies. A 34-year-old woman was diagnosed with stage I low-grade ESS at the time of hysterectomy for presumed uterine fibroma. Five years later, she recurred with multiple tumors in the lower abdomen. After an optimal surgery, she was free from progression for 6 years with progestin treatment (medroxyprogesterone acetate: MPA, 200–600 mg daily). Thereafter, she recurred twice during the MPA treatment and received debulking surgery each time. MPA was discontinued at age of 53, because another recurrent tumor grew up to 13 cm in diameter. Aromatase inhibitor anastrozole was then given at a daily dose of 1 mg with partial response (the tumor size decreased to 7 cm in diameter) for a duration of 9 months. After complete resection of the recurrent tumor, she remains progression-free for 16 months. Anastrozole was effective to recurrent low-grade ESS even after being refractory to progestin therapy. Aromatase inhibitor treatment may be a useful option as a second-line hormonal treatment to low-grade ESS.

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Keywords Low-grade endometrial stromal sarcoma · Uterine corpus · Recurrence · Aromatase inhibitor · Progestin therapy · Hormonal treatment

Introduction

Endometrial stromal sarcoma (ESS) is a rare neoplasm, accounting for 0.2% or less of gynecologic malignancies [1]. Low-grade ESS usually expresses estrogen receptors (ER) and progesterone receptors (PR), and estrogen acts as a growth stimulus [2, 3]. Objective responses have been obtained with progestin therapy, such as megestrol acetate and medroxyprogesterone acetate (MPA) [4, 5]. More recently, the efficacy of a non-steroid aromatase inhibitor has been also reported [6, 7], as it inhibits estrogen synthesis. Although either type of hormonal therapy might be useful as a first-line therapy, it is still uncertain whether a second-line hormonal treatment is effective to repetitively recurrent ESS with resistance to a first-line therapy.

We report a case of recurrent low-grade ESS with long-term survival, treated with MPA for 13 years as a first line and aromatase inhibitor anastrozole for 9 months as a second-line hormonal therapy.

Case report

A 34-year-old woman (gravida 4, para 2) underwent a total abdominal hysterectomy for presumed uterine fibroma at her local hospital in 1988. The histopathological result revealed stage I low-grade ESS of the corpus uteri. In December 1993, she was referred to our hospital, and a computed tomography (CT) scan revealed a 9-cm pelvic mass, bilateral ovarian masses (4 cm on the left and 7 cm

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on the right), and para-aortic lymph node enlargement. She underwent secondary debulking surgery, including bilateral salpingo-oophorectomy, omentectomy, bowel resection, and biopsy of para-aortic lymph nodes. All the residual tumors were less than 1 cm in diameter. The final pathology revealed recurrence of the low-grade ESS (Fig. 1a), involving the bilateral adnexae, ileum, appendix, colon, omentum, and para-aortic lymph nodes. Immunohistochemical analysis showed a strong nuclear staining for both ER and PR (Fig. 1b, c), as well as CD10 (Fig. 1d) and vimentin, and a negative staining for HHF35, 1A4, Desmin, and CD34. Postoperatively, she was started on MPA at a daily dose of 600 mg. Three years after the MPA therapy, complete response was pathologically confirmed by second look laparoscopy. MPA was continued at a daily dose of 200–400 mg without any appreciable adverse effects.

In April 2000, surgical biopsy of a 2-cm mass around the liver confirmed the recurrence of the disease on peritoneum. Two years later, she received another debulking procedure with partial liver resection for a 5-cm tumor and resection of another 5-cm pelvic tumor. After the surgery, she was hospitalized four times within 2 years due to grade 2 ileus. In June 2006, a CT scan showed a 5-cm solid mass in the left upper quadrant. The patient did not choose a debulking surgery and was kept treated with MPA at a daily dose of 200–400 mg. Eight months later, she was found to have progression of disease, represented by enlargement of the mass up to 13 cm in diameter and appearance of 4 cm mesenteric mass in the pelvis (Fig. 2a). Then, MPA treatment was discontinued, and anastrozole at a daily dose of 1 mg was started with an informed consent. After 9 months of the treatment, the tumor in the left upper quadrant was decreased to 7 cm in diameter and the mesenteric tumor was undetected (Fig. 2b). Anastrozole was discontinued because of arthritis with grade 2 joint-function disorder. Then, she underwent complete resection of the recurrent tumor. Pathological findings also revealed the significant effect of anastrozole. As shown in Fig. 3, the majority of the tumor cells was necrotic and replaced by numerous foamy histiocytes. The viable cells remained partly in the marginal lesion with expression of ER and PR. She recovered from the joint-function disorder shortly after the surgery and remains asymptomatic and progression-free for 16 months.

Discussion

ESS is subdivided histopathologically into low-grade and undifferentiated (or high-grade) forms depending on the morphology, number of mitoses, cellularity, and necrosis. The primary treatment for low-grade ESS is mainly surgery, including an abdominal hysterectomy with bilateral salpingo-oophorectomy. Adjuvant treatment, such as radiotherapy or chemotherapy, is not routinely recommended

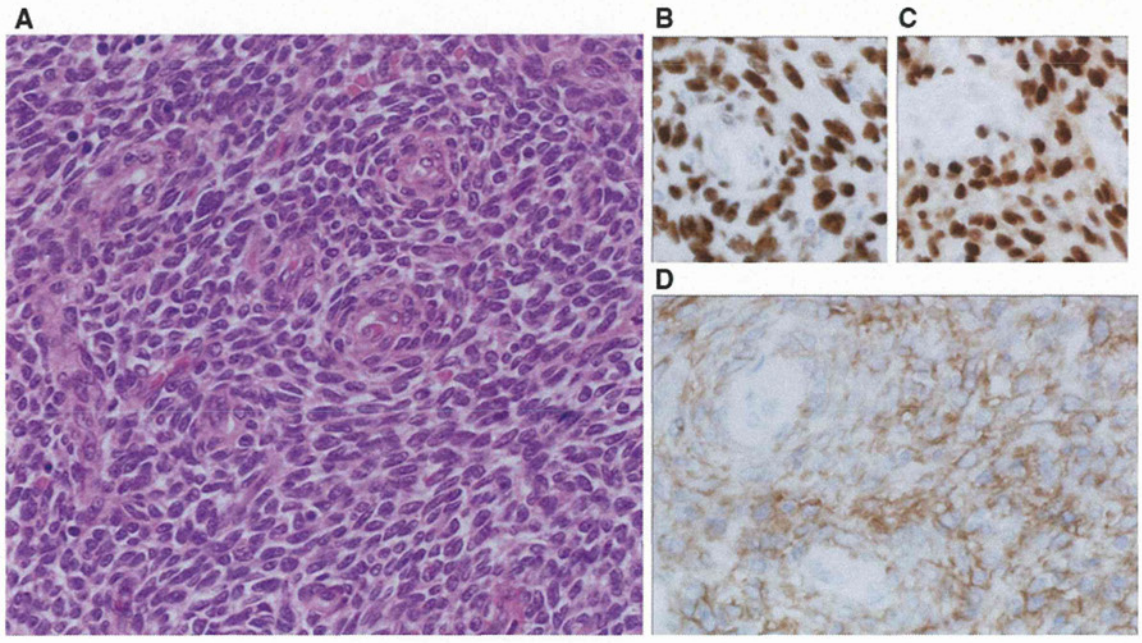

Fig. 1 Histological findings of the tumor, excised before MPA treatment. **a** High Power: Tumor cells in the pelvis, showing proliferation of endometrial stromal cells without significant atypia or pleomorphism, diagnosed as low-grade ESS. **b–d** High Power: Tumor cells are strongly positive for estrogen receptor (**b**) and progesterone receptor (**c**) and are diffusely positive for CD10 (**d**) by immunohistochemistry

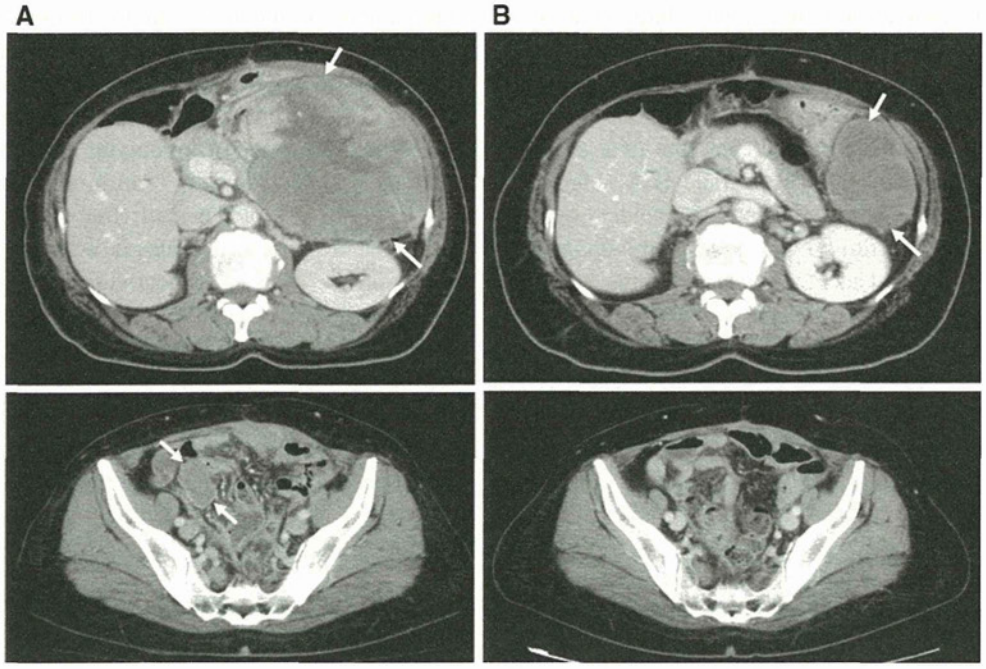

Fig. 2 Images of CT scan before and after anastrozole treatment. **a** Recurrent tumors with 13 cm in diameter in the left upper quadrant (*Upper*) and 4 cm in diameter in the pelvis (*Lower*). **b** The recurrent tumors were diminished to 7 cm in diameter (*Upper*) or became undetectable (*Lower*)

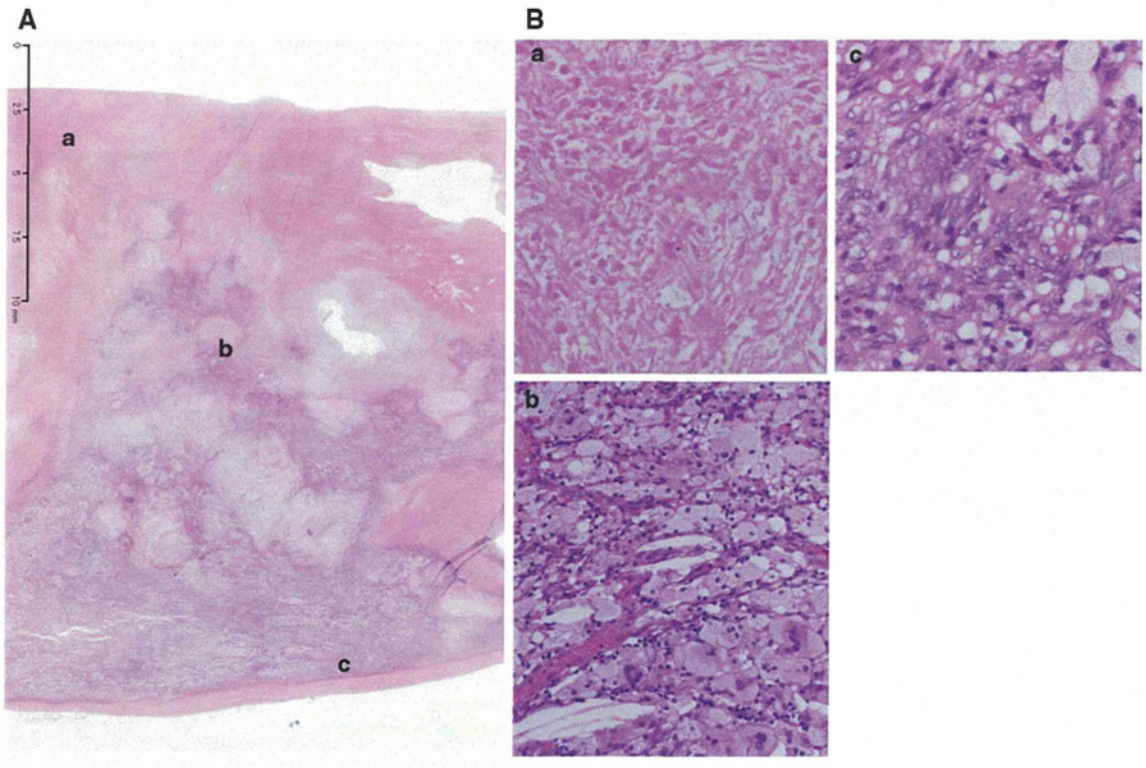

Fig. 3 Histological findings of the tumor, excised after anastrozole treatment. **a**. Low Power: Tumor cells with massive necrosis. **b** High Power: (*a*) Lesion with coagulative tumor cell necrosis, which occupies the majority of the tumor. (*b*) Center lesion with numerous foamy histiocytes. (*c*) Marginal lesion of the tumor with viable cells partly remaining

[8]. Although the prognosis of low-grade ESS is generally favorable with more than 90% of 5-year overall survival, the recurrence-free survival rate is reported to be about 50% [9, 10]. In addition to surgical resection, treatment option to recurrent low-grade ESS is hormonal therapy with progesterone derivative or aromatase inhibitor. MPA and megestrole acetate are synthetic derivatives of progesterone that exert an anti-estrogenic effect after binding to PR. The sensitivity to these progestin therapies is associated with the presence of ER and PR [11]. Aromatase inhibitors reduce estrogen levels by inhibiting its synthesis in peripheral sites. The distinct function suggests that suppressing aromatase might be still effective to recurrent ESS with resistance to progestin therapy.

The patient reported here suffered from repeated recurrences after becoming refractory to MPA treatment. Positive PR expression of the recurrent tumors suggests that the resistance to MPA therapy is caused by PR-independent manner. As a second-line hormonal therapy, anastrozole showed significant response to these recurrent tumors, suggesting that aromatase inhibitor might be useful for progestin-resistant low-grade ESS tumors. It is to be elucidated whether aromatase inhibitor is also effective to recurrent ESS tumors with negative PR expression.

Acknowledgments Conflict of interest statement All the authors declare no conflict of interest.

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CD1d, a Sentinel Molecule Bridging Innate and Adaptive Immunity, Is Downregulated by the Human Papillomavirus (HPV) E5 Protein: a Possible Mechanism for Immune Evasion by HPV▽

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CD1d and CD1d-restricted natural killer T (NKT) cells serve as a natural bridge between innate and adaptive immune responses to microbes. CD1d downregulation is utilized by a variety of microbes to evade immune detection. We demonstrate here that CD1d is downregulated in human papillomavirus (HPV)-positive cells *in vivo* and *in vitro*. CD1d immunoreactivity was strong in HPV-negative normal cervical epithelium but absent in HPV16-positive CIN1 and HPV6-positive condyloma lesions. We used two cell lines for *in vitro* assay; one was stably CD1d-transfected cells established from an HPV-negative cervical cancer cell line, C33A (C33A/CD1d), and the other was normal human vaginal keratinocyte bearing endogenous CD1d (Vag). Flow cytometry revealed that cell surface CD1d was downregulated in both C33A/CD1d and Vag cells stably transfected with HPV6 E5 and HPV16 E5. Although the steady-state levels of CD1d protein decreased in both E5-expressing cell lines compared to empty retrovirus-infected cells, CD1d mRNA levels were not affected. Confocal microscopy demonstrated that residual CD1d was not trafficked to the E5-expressing cell surface but colocalized with E5 near the endoplasmic reticulum (ER). In the ER, E5 interacted with calnexin, an ER chaperone known to mediate folding of CD1d. CD1d protein levels were rescued by the proteasome inhibitor, MG132, indicating a role for proteasome-mediated degradation in HPV-associated CD1d downregulation. Taken together, our data suggest that E5 targets CD1d to the cytosolic proteolytic pathway by inhibiting calnexin-related CD1d trafficking. Finally, CD1d-mediated production of interleukin-12 from the C33A/CD1d cells was abrogated in both E5-expressing cell lines. Decreased CD1d expression in the presence of HPV E5 may help HPV-infected cells evade protective immunological surveillance.

There are approximately 100 identified genotypes (types) of human papillomavirus (HPV). Over 40 of these are classified as genital HPV subtypes that invade the reproductive organs, including the uterine cervix, vaginal wall, vulva, and penis. Genital HPV types are further subclassified into high-risk types that are commonly associated with cervical cancer and low-risk types that cause noninvasive condyloma acuminata. Although exact classification varies among researchers, subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, and 68 are typically classified as high risk and subtypes 6, 11, 40, 42, 43, 44, 54, 61, and 72 as low risk (44). Genital HPV infection involves short-term virus proliferation, followed by the long-term latent presence of a small number of copies of the viral genome within the basal cells of the genital epithelium (44). Infections with high-risk HPV subtypes result in progression to genital tract cancers (most commonly cervical) in only a small percentage of infected women and typically after a long latency period. A high percentage of high-risk HPV DNA-positive infected women resolve their infections during the proliferative phase and thereby clear the virus or progress to latency with undetectable HPV DNA levels. The clearance of viral DNA is often accomplished through activation of the host immune system against viral antigen (19), and chronic immune suppression represents a risk factor for viral DNA persistence and benign and/or neoplastic lesion progression (23).

Completion of the HPV life cycle requires infection of epidermal or mucosal basal cells that have the potential to proliferate and differentiate. Within these cells, overall viral gene expression is suppressed, although limited expression of specific early viral genes, including E5, E6, and E7, causes lateral expansion of infected cells within the basal layer of the epithelium (44). HPV E5 seems to be particularly important early in the course of infection. Large amounts of E5 mRNA have been found in cervical intraepithelial neoplasia (CIN) lesions (37). However, as HPV-infected lesions progress to cervical cancer, episomal viral DNA becomes integrated into host cell DNA, and a substantial part of the HPV genome, commonly including the E5 coding sequence, is deleted (16). Therefore, E5 is not obligatory in the late events of HPV-mediated carcinogenesis.

E5 is a small hydrophobic protein that can be localized within the Golgi apparatus (GA), endoplasmic reticulum

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(ER), and occasionally at the plasma membrane of the host cell. It has been proposed that binding of HPV16 E5 to a subunit of the cellular proton ATPase (15) is responsible for the lack of acidification of the GA and endolysosomes and the consequent impaired function of these organelles that is seen with HPV16 infection (32, 38). The presence of HPV16 E5 has also been linked to downregulation of antigen presentation by HLA class I molecules, a process that may aid in HPVs ability to evade immune clearance through cytotoxic-T-lymphocyte (CTL)-mediated adaptive immunity (1, 2, 3, 21, 30). Ashrafi and coworkers have demonstrated that HPV16 E5 retains HLA-A and -B molecules in the GA and interferes with their trafficking to the cell surface but does not alter the transcription of HLA class I heavy chains or the transporter associated with antigen processing (TAP) (2, 3, 4, 28). Others have shown that HPV16 E5 interacts with calnexin in the ER and thereby interferes with the modification of HLA class I heavy chains (21).

CD1d is an major histocompatibility complex (MHC) class I-like glycoprotein that presents self or microbial lipid antigen to natural killer T (NKT) cells (39). In humans, a specific subset of NKT cells expresses an invariant Vα24-JaQ/Vβ11 TCR (iTCR) and can recognize CD1d on the surface of antigen-presenting cells (APCs) through this receptor. CD1d is expressed not only in typical APCs (macrophages, dendritic cells, and B cells) but also in intestinal epithelial cells (8, 12), foreskin keratinocytes (9), and reproductive tract epithelial cells (25, 26). Like MHC class I, CD1d is synthesized, glycosylated by *N*-glycosyltransferase, modified, and assembled with β2m within the ER and then transferred to the GA (5, 24, 27). CD1d plays a role in both innate and adaptive immunity to various bacteria, viruses, fungi, and parasites (reviewed in reference 10). Activation of CD1d-restricted invariant NKT (iNKT) cells enhances host resistance to some microbes in a manner that depends on the level of CD1d expression on APCs (34, 35). In contrast, the activation of iNKT cells promotes susceptibility to some microbes (7, 33). The activation of CD1d-restricted iNKT cells in response to microbial invasion is antigen dependent, but these antigens can be derived from the invading microbe or possibly from host lipids (11, 22, 29). Intracellular signaling mediated by surface CD1d utilizes NF-κB, a well-known immune-related transcription factor (36, 43). CD1d-restricted NKT cells can modulate adaptive immune cells by altering Th1/Th2 polarization. Recognition of CD1d by iNKT cells can also result in rapid release of both interleukin-4 (IL-4) and gamma interferon (IFN-γ) from the NKT cell (6). Therefore, CD1d and CD1d-restricted NKT cells serve as a natural bridge between innate and adaptive immune responses to microbes. Not surprisingly, several microbes, including herpes simplex virus type 1, human immunodeficiency virus, Kaposi's sarcoma herpesvirus, and *Chlamydia trachomatis*, are known to downregulate cell surface expression of CD1d as an immune evasion strategy (13, 26, 31, 42). Our own lab previously demonstrated that *C. trachomatis* retains CD1d in the ER and targets CD1d to both chlamydial and cellular degradation pathways (26).

Viewing the importance of CD1d in innate immune responses to microbes, we hypothesized that HPV may alter CD1d-mediated immune pathways and thereby avoid innate immune destruction of the infected cell by the host. We demonstrate here that the presence of the E5 protein of HPV6 and HPV16 is associated with reduced CD1d cell surface expression. We describe a mechanism for this downregulation and hypothesize that decreased surface CD1d expression may help HPV-infected cells evade immune surveillance during the early stages of infection.

MATERIALS AND METHODS

HPV6 and HPV16 E5 expression constructs. HPV6 and HPV16 E5 open reading frames were amplified from the HPV6 and HPV16 complete genomes (kindly provided by Tadahito Kanda, National Institute of Infectious Diseases, Japan) by PCR using primers designed to introduce BamHI (forward) and EcoRI (reverse) restriction sites. The PCR products were digested with BamHI and EcoRI and subcloned into a retroviral expression plasmid pLPCX (Clontech, Mountain View, CA).

Cell lines and establishment of a cell line stably expressing CD1d. An HPV-negative human cervical carcinoma cell line, C33A, and a vaginal epithelial cell that was originally established from normal human primary epithelial cells that were immortalized by transduction with HPV16 E6/E7 genes (VK2/E6E7) (a generous gift from D. J. Anderson, Boston University, Boston, MA) (18) were grown in Dulbecco modified eagle medium (Invitrogen, Carlsbad, CA) without $CaCl_2$ (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO_2. The vaginal epithelial (VK2/E6E7) cells used here are known to express endogenous CD1d at the cell surface (25).

A CD1d-expressing retroviral plasmid pSRα-neo (kindly provided by R. Blumberg, Harvard Medical School, Boston, MA) was transfected into Phoenix cells, a packaging cell line for recombinant retroviruses (kindly provided by K. Oda, University of Tokyo), using Lipofectamine 2000 (Invitrogen). After 72 h of incubation in DMEM, the culture medium containing released CD1d-expressing retroviruses was collected and used to infect C33A cells and transfer the CD1d gene. CD1d-expressing C33A cells were selected in medium containing 1.0 mg of neomycin/ml to establish a stably transfected cell line (C33A/CD1d).

Establishment of HPV E5-expressing cell lines. HPV6 or HPV16 E5-expressing retroviral plasmids or their empty counterparts (pLPCX-16E5, pLPCX-6E5, or pLPCX, respectively) were transfected into Phoenix cells using Lipofectamine 2000 (Invitrogen). After 72 h of incubation, culture medium with released viruses were collected and used to infect C33A/CD1d or vaginal epithelial cells. Stable cell lines were selected in media containing 1.5 μg of piromycin/ml.

Immunohistochemistry. Immunostaining for CD1d was performed on formalin-fixed, paraffin-embedded tissue sections of normal or inflamed cervix, CIN1 to CIN3, cervical cancer, and condyloma acuminata (obtained under IRB approval through the University of Tokyo). A total of 45 tissues were examined. Optimal immunostaining required antigen retrieval via microwave exposure in 0.01 M citrate buffer. A mouse anti-CD1d MAb (NOR3.2, 1:100; Abcam, Inc., Cambridge, MA) or an irrelevant, isotype-matched mouse monoclonal antibody (DakoCytomation, Glostrup, Denmark) were used as primary reagents. Immunostaining was amplified and detected by using the EnVision+System-HRP (DakoCytomation). Nuclei were counterstained by using standard hematoxylin protocols (Sigma-Aldrich, Inc., St. Louis, MO). Analyses were performed at a magnification of ×200.

Flow cytometry. C33A/CD1d cells were grown in 175-cm^2 flasks until confluent, harvested using trypsin-EDTA, and pelleted at 500 × *g* for 5 min at room temperature. The cells were then washed and resuspended in PBS-B (phosphate-buffered saline [PBS] with 1% bovine serum albumin; Invitrogen) at a concentration of 10^6 cells/ml. For detection of cell surface CD1d, 100 μl of cell suspension was incubated with an anti-CD1d NOR3.2 monoclonal antibody (MAb; Abcam) at 1:100 for 30 min at 4°C. Cells were then washed three times in PBS-B, incubated with a goat anti-mouse immunoglobulin secondary antibody conjugated to phycoerythrin (PE; BD Bioscience, San Jose, CA) for 30 min at 4°C, suspended in 1% paraformaldehyde, and analyzed by using a FACSCalibur flow cytometry system (BD Bioscience).

Proteasome inhibitor treatment. C33A/CD1d cells harboring an empty vector (C33A/CD1d-empty) or expressing HPV6 E5 (C33A/CD1d-6E5) or HPV16 E5 (C33A/CD1d-16E5) were cultured for up to 24 h in the presence or absence of the cytosolic proteasome inhibitor MG132 (10 μM in dimethyl sulfoxide [DMSO]; Sigma-Aldrich, Inc.). Control wells included vehicle alone.

HPV genotyping. DNA was extracted from cervical smear samples by using the DNeasy blood minikit (Qiagen, United Kingdom). HPV genotyping was performed by using the PGMY-CHUV assay method (20). Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and HLA-dQ primer sets (20). Reverse blotting hybridization was performed as described

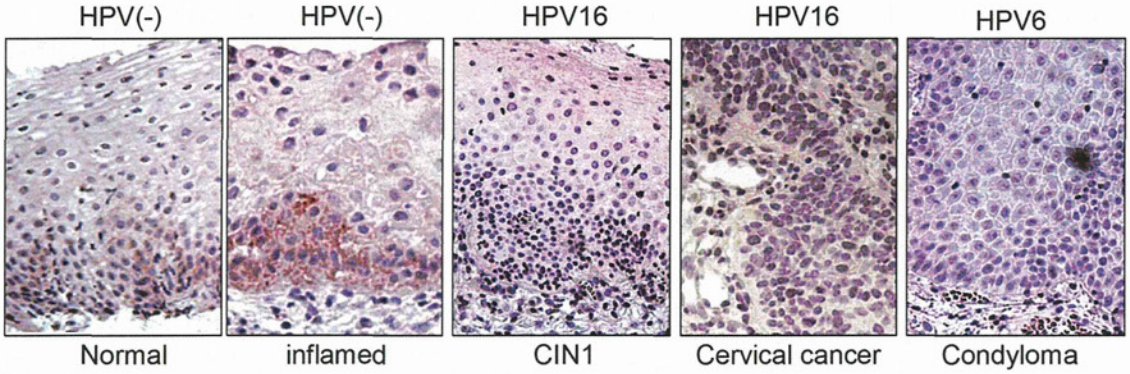

FIG. 1. Immunostaining of HPV-associated lesions for CD1d. Immunostaining for CD1d was performed after antigen retrieval on formalin-fixed, paraffin-embedded tissue sections of HPV-negative normal and inflamed ectocervical epithelium, HPV16-positive CIN1, HPV16-positive cervical cancer, and HPV6-positive condyloma acuminata. CD1d was detected with NOR3.2, a CD1d-specific MAb (1:100).

previously (20). Heat-denatured PCR amplicons were hybridized to a negatively charged nylon membrane containing specific probes for 32 HPV genotypes and HLA-dQ reference samples. Chemiluminescence detection used enhanced chemiluminescence (ECL) detection reagents for nucleic acids (GE Healthcare). Films were interpreted using the HPV reference guide provided.

RT-PCR and quantitative PCR. Portions (1 μg) of total RNA and oligo(dT)s were used for reverse transcriptase (RT) reactions (RNA PCR kit; Applied Biosystems, Foster City, CA). Total cDNA reaction samples were used as templates for amplification of each gene fragment using a PCR Core kit (Applied Biosystems). Primer pair sets for CD1d were synthesized by Invitrogen (CD1d, 453 bp; 5′-GCTGCAACCAGGACAAGTGGACGAG-3′ [forward] and 5′-AGGAACAGCAAGCACGCCAGGACT-3′ [reverse]). Those for IL-12 p40 were commercially available (Sigma-Aldrich, Inc.). For quantitative PCR, cDNA were produced via RT of 1 μg of total RNA extracted from the cells as described above by using an OmniScript RT kit (Qiagen, Inc., Valencia, CA). Portions (2 μl) of 5-fold-diluted cDNA aliquots were amplified in a thermal cycler (7300 Real-Time PCR System; Applied Biosystems) by using a QuantiTect SYBR green PCR kit (Qiagen, Inc.) and a primer pair set for β-actin (5′-GAAATCGTGCGTGACATTAAGG-3′[forward] and 5′-TCAGGCAGCTCGTAGCTTCT-3′ [reverse]). The mRNA levels for IL-12 were normalized to those of β-actin, the internal control.

Fluorescent deconvolution microscopy and confocal microscopy. C33A/CD1d cells were seeded onto coverslips. The ER was visualized using the ER tracker Blue-White DPX (Invitrogen) for 30 min at 37°C. All coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.1% Tween 20. They were then incubated for 1 h at 37°C with either an anti-CD1d NOR3.2 MAb labeled with Zenon Alexa Fluor 555 using a mouse IgG labeling kit (Invitrogen) or an anti-FLAG MAb labeled with Zenon Alexa Fluor 488 using a mouse IgG labeling kit (Invitrogen) singly or in combination. With the exception of ER tracker-treated coverslips, the cells were then counterstained with a DAPI (4′,6′-diamidino-2-phenylindole) nucleic acid stain (Invitrogen). Images were obtained with a LSM 700, flexible confocal microscope (Carl Zeiss, Oberkochen, Germany). Filter sets were optimized for Alexa 488, Alexa 555, and DAPI. Z-axis plane capture, deconvolution, and analyses were performed with ZEN 2009 Software (Carl Zeiss).

Western blotting. Portions (50 μg) of total cell lysates from C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells in a modified TNF buffer (1 M Tris-HCl [pH 7.8], 10% NP-40, 5 M NaCl, 0.5 M EDTA [pH 8.0], aprotinin, 0.1 M phenylmethylsulfonyl fluoride) were electrophoresed and transferred to nitrocellulose membranes. Membranes were blocked with 10% milk and incubated with a peroxidase-labeled anti-CD1d NOR3.2 MAb (1:200; Abcam) or an anti-FLAG MAb (1:500; Sigma-Aldrich, Inc.) using a peroxidase labeling kit (Roche, Basel, Switzerland) for 1 h. Membranes were washed and bound antibody was detected using an ECL Western blotting analysis system (GE Healthcare Buckinghamshire, United Kingdom).

Immunoprecipitation and Western immunoblotting. Harvested C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were lysed in modified radioimmunoprecipitation assay buffer (1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mM Tris, 150 mM NaCl, 2 mM EDTA) with protease inhibitors (Amersham Biosciences, Piscataway, NJ). Equivalent aliquots of total cell lysates were incubated overnight at 4C with 5 μg of mouse anti-FLAG MAbs (Sigma-Aldrich, Inc.)/ml and 5 μl of protein A-Sepharose (GE Healthcare). Precipitated proteins were separated by SDS-PAGE using 7.5% acrylamide gels and transferred to polyvinylidene difluoride membranes. Mouse anti-calnexin or rabbit anti-β-actin polyclonal antibodies (Abcam) were used as primary reagents for immunoblotting, and anti-mouse IgG-HRP (1:100,000; GE Healthcare) was used as a secondary reagent. Products in Western immunoblotting experiments were visualized by using an ECL Western blotting analysis system (GE Healthcare). Molecular masses were confirmed by comparison to standard size markers (GE Healthcare).

Statistical analysis. Quantitative PCR data were presented as means ± the standard deviations. Experiments were performed independently at least three times. The Cochran-Armitage Trend test was computed to show trends in immune reactivity with NOR3.2 MAb in clinical samples. IL-12 mRNA levels were compared to those before or after cross-linking by using paired, two-tailed Student *t* tests. A P value of <0.05 was considered significant.

RESULTS

CD1d downregulation in HPV-related lesions and cancer cell lines. Since CD1d expression in human mucosa and skin has been demonstrated by immunohistochemistry using the anti-CD1d NOR3.2 MAb (2, 9, 12, 26), we examined immunostaining of human normal ectocervix or HPV-related lesions with NOR3.2 (Fig. 1). Immunostaining for CD1d was performed on formalin-fixed, paraffin-embedded tissue sections of normal or inflamed ectocervical epithelium, cervical intraepithelial neoplasia 1 (CIN1), cervical cancer, and cervical condyloma (obtained under IRB approval through the University of Tokyo, Faculty of Medicine). To examine alterations in CD1d expression in the presence of high-risk HPV and low-risk HPV subtypes, HPV16-positive CIN1 or cancer lesions and HPV6-positive condyloma acuminata specimens were compared to each other and to HPV-negative normal and inflamed ectocervical epithelial controls. Immunoreactivity with the NOR3.2 MAb was noted in the basal and parabasal epithelial cells of normal and inflamed ectocervical epithelia that are known to express early HPV genes (E5, E6, and E7; Fig. 1) (44). In inflamed epithelium, the immunoreactivity appeared to be intensified compared to normal epithelium. CD1d expression is known to be enhanced by inflammatory cytokines (10). NOR3.2 immunoreactivity is essentially absent in HPV16-positive CIN1, HPV16-positive cancer, and HPV6-positive condyloma lesions (Fig. 1). To statistically analyze alterations in CD1d expression, a total of 45 clinical specimens from normal controls and HPV-related lesions were immunostained with NOR3.2 (Table 1). NOR3.2 immunoreactivity was

TABLE 1. Immunoreactivity with NOR3.2 anti-CD1d MAb in cervical epithelium of various lesions

Histological status	HPV status[a]	CD1d (no. of cases)		% Positive[b]
		Positive	Negative	
Normal/inflamed		9	1	90.0
CIN1 and CIN2	HR-HPV(+)	0	7	0
CIN3	HR-HPV(+)	2	16	11.1
Cancer	HR-HPV(+)	0	7	0
Condyloma	HPV6(+)	0	3	0

[a] HR-HPV(+), any high-risk HPV positive.
[b] $P = 0.0001$ (exact Cochran-Armitage Trend test).

mostly limited to the HPV-negative normal or inflamed ectocervical epithelial samples similar to those represented in the first two panels of Fig. 1. NOR3.2 immunoreactivity was absent in all CIN1 and CIN2, cervical cancer, and condyloma lesions. Among CIN3 samples, two lesions showed NOR3.2 immunoreactivity, whereas 16 lesions did not. Using trend analysis, we were able to demonstrate an association between decreased CD1d immunoreactivity and progression of cervical neoplastic lesions with statistical significance ($P = 0.0001$).

Although HPV E5 is not expressed in cervical cancer cells (16), immunohistochemical data demonstrated that CD1d expression was also abrogated in cervical cancer lesions. To address the mechanisms underlying CD1d downregulation in cervical cancers, we examined the level of CD1d transcription and CD1d expression at the cell surface in several cervical cancer cell lines (Fig. 2). As a positive control, we created cell transfectants that stably expressed CD1d. To avoid the potential influence of endogenous HPV protein expression, an HPV-negative cervical cancer cell line, C33A, was used for our CD1d transfectants. We used a retrovirus vector to transduce the CD1d gene into these cells and established the stable cell line, C33A/CD1d via neomycin selection. Flow cytometry revealed strong expression of CD1d on the cell surface of C33A/CD1d cells. Cd1d was not expressed on the cell surface of C33A control cells or in other cancer cell lines (Fig. 2A). To examine the level of CD1d transcription in these same cells, cDNA was produced via RT of total RNA from each cell line and subjected to PCR using primer pairs specific for CD1d. The expected single band representing CD1d was observed on agarose gels only in C33A/CD1d cells (Fig. 2B). These data indicated that CD1d expression was abrogated prior to or during transcription the tested cervical cancer cell lines.

Cell surface expression of CD1d decreases in HPV E5-expressing epithelial cells. HPV E5 has been reported to inhibit cell surface expression of HLA class I molecules by interfering with their trafficking to the cell surface (1, 2, 3, 21, 30). Since CD1d and HLA class I heavy chains utilize an identical intracellular pathway to traffic from the ER to the cell surface, we hypothesized that HPV E5 may also interfere with surface CD1d expression at a posttranscriptional level. To verify our immunohistochemical data and study CD1d trafficking in the presence of E5 *in vitro*, we created HPV6 and HPV16 E5 stably transfected cell lines using C33A/CD1d cells. Since the E5 protein is less than 10 kDa in size, the production of an anti-E5 antibody would be difficult. Instead, E5 proteins were tagged with FLAG and detected by Western blotting or immunostaining with an anti-FLAG antibody. FLAG-tagged HPV6 or HPV16 E5 genes were transduced into the C33A/CD1d cells by using retrovirus vectors. To control for the influence of retrovirus infection and the presence of the expression vector, C33A/CD1d cells were infected with empty retrovirus vectors. Retrovirus-infected cells were exposed to puromycin, and E5-expressing C33A/CD1d cells were established (C33A/CD1d-6E5, -16E5, and -empty). In Fig. 3A, lanes 5 and 6, show PCR products derived from cDNA generated by RT of total RNA from C33A/CD1d-6E5 and -16E5 cells. Lanes 2 and 3 in the same figure show PCR products derived from corresponding expression plasmid DNA. FLAG-6E5 and -16E5 were transcribed in C33A/CD1d-6E5 and -16E5 cells, respectively. Using Western immunoblotting and an anti-FLAG MAb, FLAG-6E5 and -16E5 proteins were detected as immunoreactive bands at an approximate size of 10 kDa in lanes 1 and 2, respectively (Fig. 3B).

We next examined the expression of CD1d at both mRNA and protein levels in the presence or absence of HPV E5. CD1d transcription levels in C33A/CD1d cells were unaffected by the presence of E5 or of empty vector compared to naive

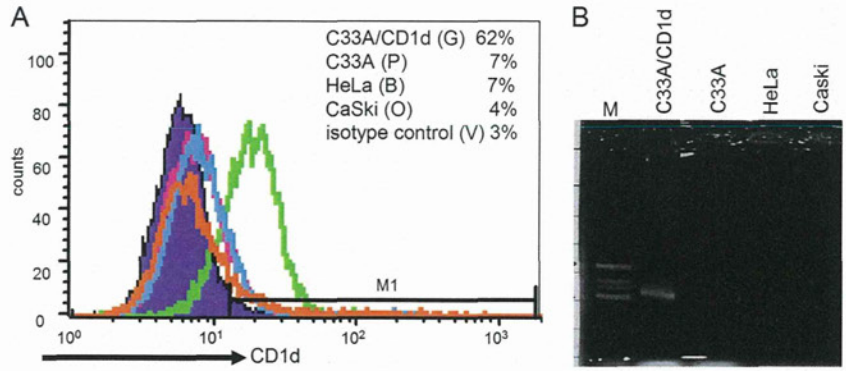

FIG. 2. CD1d alterations in cancer cell lines. (A) Cell surface expression of CD1d in C33A (pink line), HeLa (blue line), CaSki (orange line), and C33A/CD1d (green line) cells. All cells were stained with an anti-CD1d primary MAb (NOR3.2; 1:100 dilution) and a PE-conjugated goat anti-mouse immunoglobulin secondary antibody (1:20 dilution). Background staining of the cells using an isotype-matched control antibody is also shown (filled region). Cells were suspended in 1% paraformaldehyde and analyzed by using a FACSCalibur flow cytometry system. (B) Transcription of CD1d. cDNA was produced via RT of 1 μg of total RNA from each cell line and amplified by PCR with primer pairs specific for CD1d. PCR products were separated over an agarose gel containing ethidium bromide.

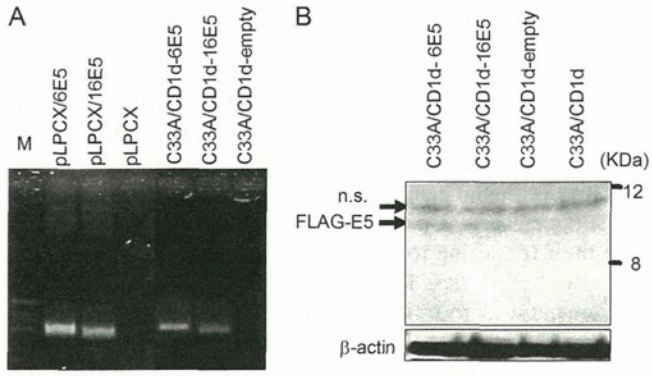

FIG. 3. HPV E5 detection in HPV E5-transformed C33A/CD1d cells. (A) Transcription of HPV E5. cDNA was produced via RT of 1 μg of total RNA from each cell line and amplified by PCR with primer pairs specific for HPV16 E5 and HPV6 E5. PCR products were separated over an agarose gel containing ethidium bromide. Lanes 5 and 6 display PCR products derived from C33A/CD1d-6E5 and -16E5 cDNA, respectively, while lanes 2 and 3 show PCR products derived from corresponding expression plasmid DNA. Lanes 4 and 7 represent negative control plasmid and cell lines lacking E5, respectively. (B) Translation of HPV E5. Fifty-microgram aliquots of protein lysates from each cell line were analyzed by Western immunoblotting with antibodies against the FLAG tag (1:500 dilution) and β-actin (loading control).

C33A/CD1d cells (Fig. 4A). In contrast, the 48-kDa, mature glycosylated form of the CD1d heavy chain (HC) that was detected in naive C33A/CD1d and C33A/CD1d-empty cells was completely abrogated in C33A/CD1d-6E5 and barely detectable in the C33A/CD1d-16E5 cells (Fig. 4B, lanes 1, 4, 2, and 3, respectively). The presence of HPV6 and HPV16 E5 drastically inhibited the maturation of CD1d HCs. Flow cytometry was used to analyze the effect of HPV E5 on cell surface expression of CD1d in the C33A/CD1d cells harboring E5-expressing or empty vector (Fig. 5). CD1d was expressed by

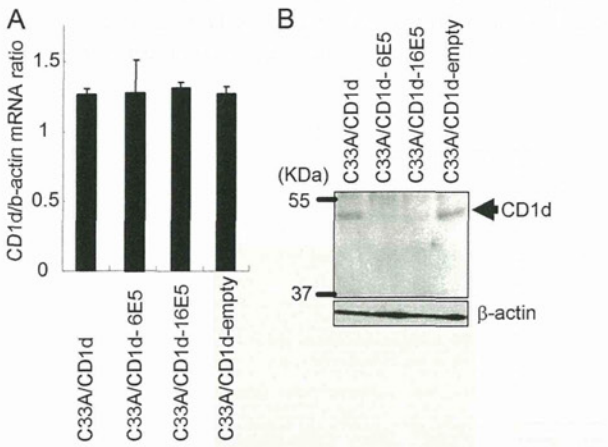

FIG. 4. CD1d heavy-chain transcription and translation in C33A/CD1d, C33A/CD1d-empty, C33A/CD1d-6E5, and C33A/CD1d-16E5 cells. (A) Transcription of CD1d HC. The mRNA levels of CD1d were analyzed by quantitative RT-PCR using SYBR green methodology. CD1d mRNA levels were normalized to β-actin. (B) Fifty-microgram aliquots of protein lysates from each cell line were analyzed by Western immunoblotting with a peroxidase-labeled anti-CD1d NOR3.2 MAb (1:200 dilution) and a β-actin loading control.

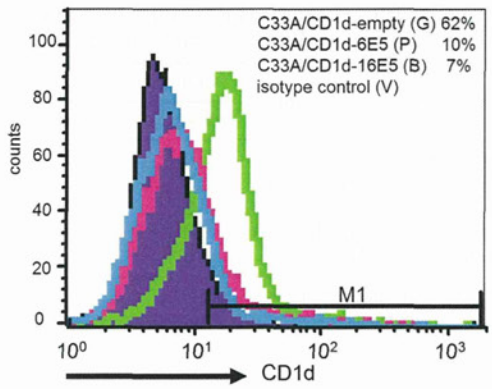

FIG. 5. Cell surface expression of CD1d in C33A/CD1d-empty (green line), C33A/CD1d-6E5 (pink line), and -16E5 (blue line) cells. All cells were stained with an anti-CD1d primary MAb (NOR3.2; 1:100 dilution) and a PE-conjugated goat anti-mouse immunoglobulin secondary antibody (1:20 dilution). Background staining of the cells with an isotype-matched control antibody is also shown (filled region). Cells were suspended in 1% paraformaldehyde and analyzed using a FACSCalibur flow cytometry system.

the majority of C33A/CD1d-empty cells but absent in >70% of C33A/CD1d-6E5 or -16E5 cells (Fig. 5).

To confirm the effect of E5 on endogenous CD1d, we used a vaginal epithelial cell line immortalized via HPV16 E6/E7 transduction of primary cells collected from normal human vaginal epithelium and subsequently well characterized as possessing histological and immunological characteristics identical to those of primary epithelial cells (18). We have previously reported the endogenous expression of functional CD1d molecules on the surface of these cells (25). Since vaginal epithelial cells are well-known targets of genital HPV, these cells were considered to be useful as an *in vitro* model for *in vivo* HPV infections. FLAG-tagged HPV6 or HPV16 E5 genes were transduced into these vaginal cells by using retrovirus vectors (Vag-6E5 and -16E5). We then examined the expression of CD1d at various levels in the presence or absence of HPV E5 (Fig. 6). RT-PCR and Western blotting revealed that CD1d transcription was unaffected by the presence of E5, but the 48-kDa CD1d HC product clearly decreased in Vag-6E5 and -16E5 cells compared to naive and Vag-empty cells (Fig. 6A). Flow cytometry confirmed the decreased cell surface expression of CD1d in 6E5-expressing vaginal epithelial cells (Fig. 6B).

E5-expressing epithelial cells retain CD1d in the ER. To demonstrate the intracellular localization of CD1d heavy chains in C33A/CD1d cells harboring HPV-6E5 and -16E5, immunofluorescence confocal microscopy was performed with an anti-CD1d MAb (NOR3.2) combined with either an anti-FLAG MAb that detects FLAG-E5 proteins, an ER-specific marker (ER tracker) or DAPI (Fig. 7). In C33A/CD1d-empty control cells, dual labeling for CD1d and the nucleus (DAPI) verified that CD1d could be detected in a diffuse pattern throughout the intracellular space, with increased accumulation near the cell surface but not in the perinuclear area (Fig. 7, upper image). In contrast, decreased amounts of CD1d could be detected in C33A/CD1d-6E5 and -16E5 cells and CD1d proteins were localized to perinuclear areas near the ER. CD1d and ER signals merged in perinuclear areas (pink

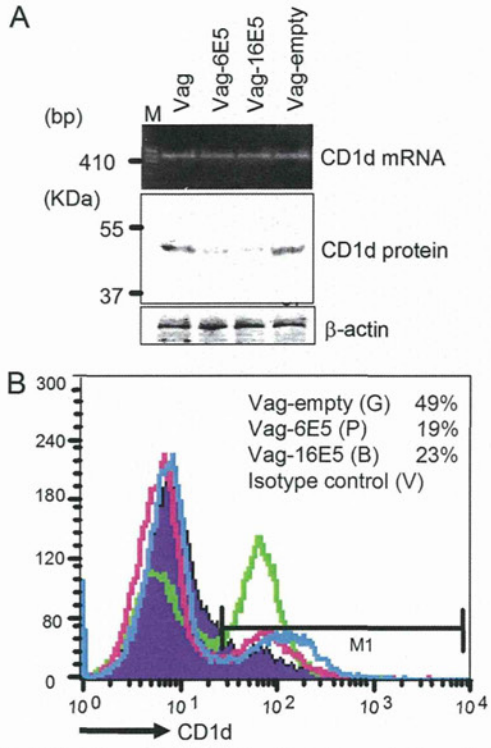

FIG. 6. CD1d downregulation in alternate genital keratinocytes in the presence of 6E5 and 16E5. (A) HPV6 and HPV16 E5 genes were transduced into vaginal epithelial cells established from normal human vaginal epithelium (17) and named Vag-6E5 and Vag-16E5, respectively. PCR products derived from cDNA generated by reverse transcription using 1 μg of total RNA from each of the vaginal cell lines were separated over an ethidium bromide-containing agarose gel. Fifty-microgram aliquots of protein lysates from each vaginal cell line were analyzed by Western immunoblotting with a peroxidase labeled anti-CD1d NOR3.2 MAb (1:200 dilution) and a β-actin loading control. (B) Vag-empty (green line), Vag-6E5 (pink line), and Vag-16E5 (blue line) were stained with an anti-CD1d primary MAb (NOR3.2; 1:100 dilution) and a PE-conjugated goat anti-mouse immunoglobulin secondary antibody (1:20 dilution). Background staining of the cells using an isotype-matched control antibody is also shown (filled region). Cells were suspended in 1% paraformaldehyde and analyzed by using a FACSCalibur flow cytometry system.

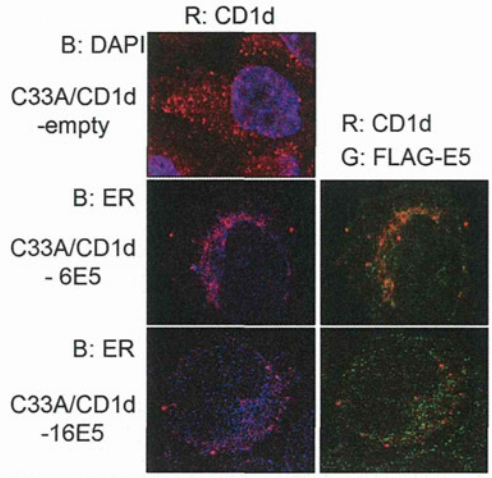

FIG. 7. CD1d trafficking in the presence or absence of E5. C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were seeded onto coverslips. All of the cells were immunostained with an anti-CD1d MAb (NOR3.2, red). C33A/CD1d-empty were also exposed to DAPI (blue), and C33A/CD1d-6E5 or -16E5 cells were exposed to ER tracker (blue) and an anti-FLAG MAb (green). Cells were then visualized by using fluorescence confocal microscopy. Orange to yellow signals represent colocalization of CD1d and E5 within the ER.

signals), suggesting that the majority of CD1d is within the ER (Fig. 7, images on the left). Dual labeling for CD1d and FLAG-E5 verified the colocalization of CD1d and E5 within the ER (orange to yellow signals), while nonmerged FLAG-E5 signals were present in the perinuclear area (pure green), suggesting the presence of E5 in the GA in the absence of CD1d (Fig. 7, images on the right). The results of immunofluorescence microscopy support our biochemical and flow cytometry data showing that mature CD1d protein levels decrease and CD1d fails to traffic to the cell surface in HPV E5-expressing cells.

HPV E5 interacts with calnexin in the ER. Previous biochemical studies have reported that HPV16 E5 interacts with calnexin and that these interactions interfere with the modification of HLA class I heavy chains that typically occurs in the ER (21). The role of calnexin and/or calreticulin in the formation of the second disulfide bond of CD1d HCs in the ER is well described (24). We therefore hypothesized that E5 interacts with calnexin in the ER and may impair calnexin-mediated CD1d folding. This, in turn, could interrupt appropriate trafficking of CD1d to the surface of HPV-infected cells. To address the hypothesis, we examined the interaction of E5 with calnexin using immunoprecipitation. Total cell lysates obtained from C33A/CD1d-empty, -6E5, and -16 E5 cells were incubated with anti-FLAG MAb conjugated beads. FLAG-E5-bound proteins were immunoprecipitated and analyzed by immunoblotting with an anti-calnexin MAb. A band with an apparent molecular mass of 90 kDa and corresponding to calnexin was detected in C33A/CD1d-6E5 and -16 E5 cells, but not C33A/CD1d-empty cells, biochemically demonstrating interaction between E5 with calnexin (Fig. 8A).

To visually demonstrate the colocalization of CD1d and calnexin, C33A/CD1d-empty, -6E5, and -16E5 cells were dually stained with anti-CD1d NOR3.2 and anti-calnexin MAbs and examined by using confocal microscopy. Again, NOR3.2-reactive CD1d was detected throughout the intracellular space in C33A/CD1d-empty cells. In contrast, the majority of CD1d molecules in C33A/CD1d-6E5 or -16E5 cells localized to the perinuclear area (Fig. 8B, images on left). Calnexin detection was rendered as green signals. These mostly localized to perinuclear areas in E5-expressing cells and correspond to the location of ER (Fig. 8B, center images). Although some merge images (yellow signals) could be detected in each cell line, the merge patterns differed between C33A/CD1d-empty and E5-expressing cells (Fig. 8B, images on the right). In C33A/CD1d-empty cells, the calnexin and CD1d signals were mostly distinct and but those that did colocalize appeared to follow the synthetic pathway for type I proteins. In contrast, CD1d in the E5-expressing cells completely colocalized with calnexin, confirming our biochemical data demonstrating physiologic interaction between calnexin and CD1d in the C33A/CD1d-6E5 and -16E5 cells.