Int J Clin Oncol
DOI 10.1007/s10147-011-0280-9

ORIGINAL ARTICLE

Subsequent risks for cervical precancer and cancer in women with low-grade squamous intraepithelial lesions unconfirmed by colposcopy-directed biopsy: results from a multicenter, prospective, cohort study

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Received: 29 March 2011 / Accepted: 15 June 2011
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Abstract
Objective To investigate the natural course of low-grade squamous intraepithelial lesions (LSILs) that cannot be histologically confirmed by colposcopy-directed biopsy.
Methods In a multicenter, prospective, cohort study of Japanese women with LSILs, we analyzed the follow-up data from 64 women who had a negative biopsy result at the initial colposcopy (biopsy-negative LSIL) in comparison with those from 479 women who had a histologic diagnosis of cervical intraepithelial neoplasia grade 1 (LSIL/CIN1). Patients were monitored by cytology and colposcopy every 4 months for a mean follow-up period of 39.0 months, with cytologic regression defined as two consecutive negative smears and normal colposcopy.
Results In women with biopsy-negative LSILs, there were no cases of CIN3 or worse (CIN3+) diagnosed within 2 years; the difference in the 2-year risk of CIN3+ between the two groups was marginally significant (0 vs. 5.5%; $P = 0.07$). The cumulative probability of cytologic regression within 12 months was much higher in the biopsy-

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Published online: 13 July 2011

negative LSIL group (71.2 vs. 48.6%; $P = 0.0001$). The percentage of women positive for high-risk human papillomaviruses (hrHPVs) was significantly lower in the biopsy-negative LSIL group than in the LSIL/CIN1 group (62.1 vs. 78.4%; $P = 0.01$); however, the 12-month regression rate of biopsy-negative LSIL was similar between hrHPV-positive and -negative women (67.3 vs. 74.4%, $P = 0.73$).
Conclusion In women with biopsy-negative LSILs, the risk of CIN3+ diagnosed within 2 years was low; furthermore, approximately 70% underwent cytologic regression within 12 months, regardless of HPV testing results. Biopsy-negative LSILs may represent regressing lesions rather than lesions missed by colposcopy.

Keywords Low-grade squamous intraepithelial lesion · Colposcopy · Human papillomavirus · Cervical intraepithelial neoplasia

Introduction

In the Bethesda System for cytologic reporting, a low-grade squamous intraepithelial lesion (LSIL) represents mild cervical abnormalities, including cellular changes associated with human papillomavirus (HPV) infection and cervical intraepithelial lesion grade 1 (CIN1) [1]. However, approximately 15–20% of women with a cytologic interpretation of LSIL have a grade 2 (CIN2) or grade 3 (CIN3) cervical intraepithelial lesion, which are immediately treated with cervical ablation, loop electrosurgical excision procedure (LEEP) or cone biopsy [2, 3]. Therefore, women with LSILs usually undergo a colposcopy-directed biopsy for histologic evaluation of cervical abnormalities. Of the women with LSIL cytology, 40–60% are found to have a histologic diagnosis of CIN1 at the initial colposcopy, while 15–30% have a negative biopsy result [2, 3]. According to the 2006 American Society for Colposcopy and Cervical Pathology consensus management guidelines [4], the follow-up strategy for women with a negative biopsy result is identical to that of women with CIN1; that is, both groups are followed with either repeated cytology at 6 and 12 months or HPV testing at 12 months. However, the natural course of LSILs that cannot be histologically diagnosed by colposcopy-directed biopsy has not been well documented.

The Japan HPV and Cervical Cancer (JHACC) cohort study was designed to identify determinants of regression and progression of low-grade cervical abnormalities [5, 6]. In the primary analysis, we used only the follow-up data from 570 women with cytologic LSIL and histologically confirmed CIN1 or CIN2 lesions, and demonstrated HPV type-specific risks of LSIL persistence and progression [5]. In the present study, we analyzed the follow-up data of 64 women with biopsy-negative LSIL who were excluded from the main analysis cohort.

Methods

Study design

This study represents a secondary analysis of data from the prospective non-intervention cohort study conducted by the JHACC study group for identifying determinants of LSIL/CIN regression and progression. Details of the design, methods, and primary results have been provided in more detail elsewhere [5, 6]. Briefly, 905 women with mildly abnormal cytology were recruited from nine hospitals that performed conventional Pap smears, colposcopy and cervical biopsies. The inclusion criteria of this secondary analysis were: evident LSIL cytology; histologic diagnosis of CIN1 or less at initial colposcopy and biopsy; age 18–54 years; first detection of cervical abnormality; and a sufficient number (two or more) of follow-up visits. Women entered the study voluntarily after giving their signed informed consent. Cervical smears were classified according to the Bethesda System [1]. At the time of study entry, two (or more) small cervical specimens were taken by colposcopy-directed punch biopsy and stained with hematoxylin and eosin (H&E). A histologic diagnosis was determined based on the World Health Organization (WHO) classification system. Two cytopathologists (Y.H. and Masafumi Tsuzuku) and two pathologists (R.F. and T.K.) reviewed all cytologic and histologic specimens collected at the time of entry. Patients were tested for cervical HPV DNA, serum IgG antibodies to cytomegalovirus (CMV), *Chlamydia trachomatis*, and herpes simplex virus type 2 (HSV2) at the time of entry. The researchers who performed the assays were blinded to the clinical data collected from the study subjects. Information regarding smoking and sexual behavior was obtained from a self-administered questionnaire. Patients were routinely followed at 3- to 4-month intervals and received cytologic and colposcopic examinations at each visit. To avoid interference from the biopsy procedure on the natural course of the disease, a cervical biopsy was performed during the follow-up period only when Pap smears and colposcopic findings were suggestive of the presence of CIN3 or worse (CIN3+). A cytology result of HSIL triggered colposcopy-guided biopsy during follow-up examinations. The two cytopathologists and the two pathologists reviewed all cytologic and histologic specimens collected for the diagnosis of CIN3+. We chose an end-point of CIN3 or cancer rather than CIN2 or higher because CIN2 likely represents a heterogenous collection of cervical abnormalities [7, 8], only some of which progress to CIN3 [5, 9]. In this analysis, we defined regression as normal colposcopy results and at least two consecutive negative Pap smears. Persistent lesions were defined as lesions that did not regress or were diagnosed with CIN3+ during the follow-up period.

Overall, the study subjects consisted of 554 women who had a negative biopsy result (biopsy-negative LSIL; $n = 64$) or a histologic diagnosis of CIN1 (LSIL/CIN1; $n = 491$) at the initial colposcopy for LSIL cytology. Unfortunately, data from cervical samples, blood samples, or questionnaires were not available in all 554 study subjects. Cervical HPV data were not available in 21 women because of insufficient samples, while data on serum antibodies to sexually transmitted agents were lacking in 23 women. In addition, 54 women gave no responses to a self-administrated questionnaire. The study protocol was approved by the ethical and research review boards of the participating institutions.

HPV genotyping

We detected HPV DNA in cervical samples by polymerase chain reaction (PCR)-based methodology, as previously described [10]. In brief, exfoliated cells from the ectocervix and endocervix were collected in a tube containing 1 ml of phosphate-buffered saline (PBS) and stored at −30°C until DNA extraction. Total cellular DNA was extracted from cervical samples by a standard sodium dodecyl sulfate (SDS)–proteinase K procedure. HPV DNA was PCR amplified by 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. Primers for a fragment of the β-actin gene were also used as a control to rule out false-negative results for samples in which HPV DNA was not detected. HPV types were identified by an analysis of restriction fragment length polymorphism (RFLP), which has been shown to identify at least 26 types of genital HPVs [11].

IgG antibody against sexually transmitted agents

The level of IgG antibodies to *Chlamydia trachomatis* and HSV2 was determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits: *Chlamydia trachomatis* (HITAZYME; Hitachi Chemical, Tokyo, Japan) and HSV2 (HerpeSelect 2 ELISA IgG; Focus Diagnostics, Cypress, CA, USA). The serologic assay for *Chlamydia trachomatis* utilizes purified EB outer-membrane proteins of the *Chlamydia trachomatis* L2 strain as antigens and does not detect antibody to *Chlamydia pneumoniae* [12]. These serologic assays were performed at a clinical testing laboratory (SRL, Tokyo, Japan).

Statistical analysis

All time-to-event analyses were based on the actual date of the 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 cytologic transition to normal occurred or CIN3+ was first detected. Women whose lesions persisted or who dropped out of the study were censored at their last recorded return visit dates. Subjects 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 LSIL regression or progression was estimated by using the Kaplan–Meier method and compared with a log-rank test. All analyses were carried out using the JMP 7.0J (SAS Institute, Cary, NC, USA) statistics packages. Two-sided P values were calculated throughout and considered to be significant at less than 0.05.

Results

We analyzed the follow-up data from a total of 554 women with LSIL cytology who had a negative biopsy result (biopsy-negative LSIL; $n = 64$) or a histologic diagnosis of CIN1 (LSIL/CIN1; $n = 491$) at the initial colposcopy. Distributions of baseline characteristics between these two groups are presented in Table 1. The women with biopsy-negative LSILs were older than the women with LSIL/CIN1 (mean age ± SD 38.8 ± 9.2 vs. 36.2 ± 7.7 years); however, the difference in the age distribution between the two groups was only marginally significant ($P = 0.07$). Cervical HPV infections were found in 75.0% of women with biopsy-negative LSILs and in 84.6% of women with LSIL/CIN1 results and the difference was statistically significant ($P = 0.02$). The percentage of women positive for high-risk human papillomaviruses (hrHPVs) was also significantly lower in the biopsy-negative LSIL group than in the LSIL/CIN1 group (62.1 vs. 78.4%; $P = 0.01$). The percentage of women who had smoked was lower in the biopsy-negative LSIL group (32.6 vs. 48.7%), but the difference was only marginally significant ($P = 0.07$). The number of lifetime sexual partners was significantly greater among women with LSIL/CIN1 than among women with biopsy-negative LSILs ($P = 0.001$). The age at first sexual intercourse was also lower among women with LSIL/CIN1 compared to women with biopsy-negative LSILs, although the difference was only marginally significant ($P = 0.06$). Women with LSIL/CIN1 were likely to have a higher IgG antibody titer against *Chlamydia trachomatis* than women with biopsy-negative LSILs; however, the difference was not significant ($P = 0.25$). The IgG reactivity to HSV2 was similar between the two groups ($P = 0.82$). At least two

Table 1 Characteristics of the study subjects

	Cytology and histology		P values[†]
	Biopsy-negative LSIL ($n = 64$)[a]	LSIL/CIN1 ($n = 479$)	
Age (years)			
Mean (SD)	38.8 (9.2)	36.2 (7.7)	
18–29	11 (17.2%)	95 (19.8%)	0.07
30–39	21 (32.8%)	215 (44.9%)	
40+	32 (50.0%)	169 (35.3%)	
HPV typing			
Positive for high-risk types[b]	36 (62.1%)	359 (78.0)	0.01
Negative for high-risk types	22 (37.9%)	101 (22.0%)	
Positive for any HPV	48 (77.4%)	405 (88.0%)	0.02
Negative for any HPV	14 (22.6%)	55 (12.0%)	
Smoking			
Never smokers	37 (63.8%)	222 (51.3%)	0.07
Smokers	21 (36.2%)	211 (48.7%)	
Current smokers	16 (27.6%)	143 (33.0%)	
Former smokers	5 (8.6%)	68 (15.7%)	
Number of lifetime sexual partners			
1	23 (39.6%)	79 (18.1%)	0.001
2–3	13 (22.4%)	129 (29.5%)	
4+	22 (37.9%)	229 (52.4%)	
Age at first sexual intercourse (years)			
≤20	12 (20.3%)	147 (34.2%)	0.06
21–23	26 (44.1%)	179 (41.6%)	
≥24	21 (35.6%)	104 (24.2%)	
IgG antibodies to *Chlamydia trachomatis*			
Low	27 (45.0%)	166 (36.1%)	0.25
Mid	20 (33.3%)	150 (32.6%)	
High	13 (21.7%)	144 (31.3%)	
IgG antibodies to HSV2			
Low	23 (38.3%)	158 (34.3%)	0.82
Mid	19 (31.6%)	150 (32.6%)	
High	18 (30.0%)	152 (33.0%)	

[†] These P value were calculated by the χ^2 test

[a] Biopsy-negative LSIL denotes women with LSILs that had a negative biopsy result at the initial colposcopy

[b] HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 were classified into high-risk HPV types

biopsies were taken at the initial colposcopy and there was no difference in the number of biopsies between the two groups.

Patients were monitored by cytologic and colposcopic testing at intervals of 3–4 months. Among women with biopsy-negative LSILs, no case was diagnosed with CIN3+ within 2 years; the difference in the cumulative risk of CIN3+ diagnosed within the next 2 years between the two groups was marginally significant (0 vs. 5.5%; $P = 0.07$ by log-rank test; Fig. 1a). In women with biopsy-negative LSILs, the majority of cytologic regression occurred within 12 months. The cumulative probability of cytologic regressions within 12 months was much higher in women with biopsy-negative LSILs than in women with LSIL/CIN1 (71.2 vs. 48.6%; $P = 0.0001$; Fig. 1b). The 2-year rate of cytologic regression was also significantly different between the two groups (75.1 vs. 64.0%; $P = 0.003$). Cytologic regression occurred more quickly in women with biopsy-negative LSILs than in women with LSIL/CIN1 (median time to regression: 6.3 vs. 12.4 months). In the women with biopsy-negative LSILs, the 12-month cumulative probability of cytologic regression was similar between hrHPV-positive and -negative women (67.3 vs. 74.4%; $P = 0.74$); median time to regression was also similar between hrHPV-positive and -negative women (5.4 vs. 7.7 months; $P = 0.45$; Fig. 2a). In women with LSIL/CIN1, however, detection of hrHPVs significantly influenced the 12-month rate of cytologic regression (hrHPV-positive [45.2%] vs. hrHPV-negative [62.6%]; $P = 0.006$; Fig. 2b).

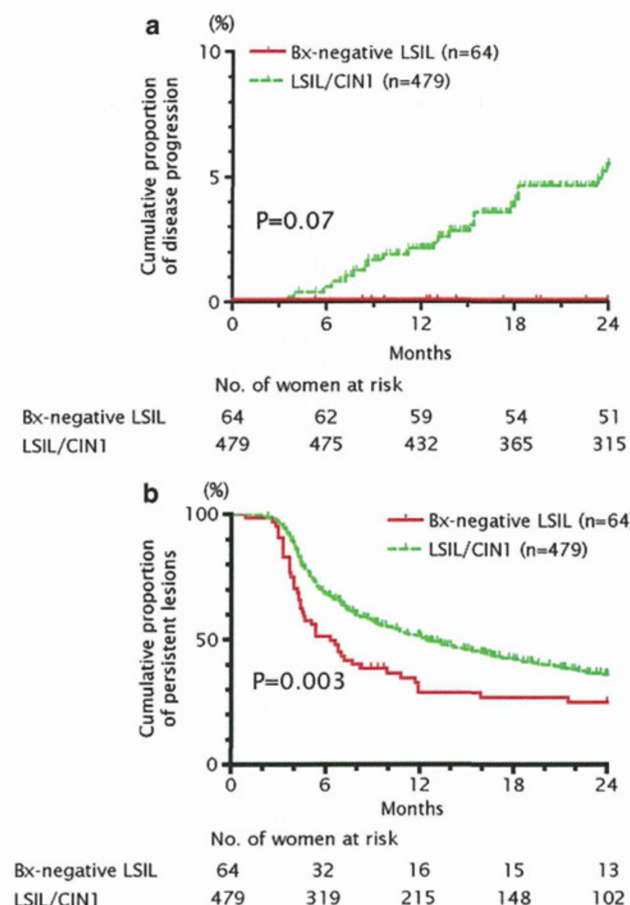

Fig. 1 Cumulative probabilities of CIN3+ diagnosis and cytologic regression within 2 years. A Kaplan–Meier plot was used to estimate the cumulative probabilities of CIN3+ diagnosis (**a**) and cytologic regression (**b**) within 2 years among women with biopsy-negative LSILs (*solid line*) or LSIL/CIN1 (*dashed line*). *P* values were calculated by the log-rank test

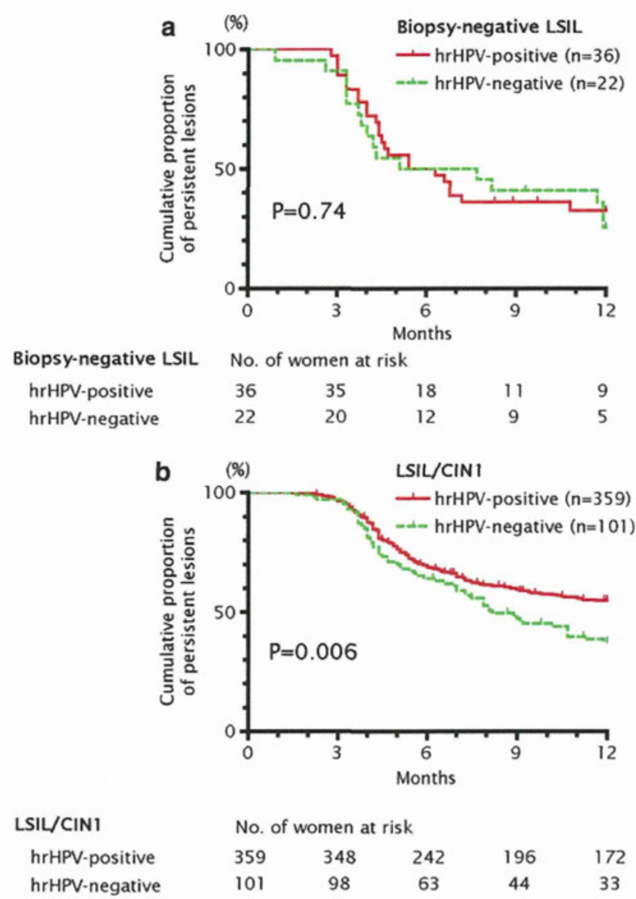

Fig. 2 Cumulative probabilities of cytologic regression within 12 months according to detection of hrHPVs. A Kaplan–Meier plot was used to estimate the cumulative probabilities of cytologic regression within 12 months among women with biopsy-negative LSILs (**a**) or LSIL/CIN1 (**b**) according to hrHPV detection. *P* values were calculated by the log-rank test

Discussion

Colposcopy-directed biopsies are recommended for women with LSIL cytology, primarily to exclude a high-grade lesion. Although approximately 15–30% of those women have a negative biopsy result [2, 3], they are routinely subjected to follow-up because of uncertainty about the risk of precancerous lesions missed by a colposcopic biopsy. In the present study, women with a biopsy-negative LSIL (i.e., "unconfirmed" LSIL) were at substantially low risk of CIN3 or cancer diagnosed within the following 2 years. The women with biopsy-negative LSILs were also significantly more likely to have cytologic regression than women with LSILs underlying CIN1. Some cases of biopsy-negative LSIL may be based on false-positive cytology because the percentage of women negative for any HPV was significantly higher in the biopsy-negative LSIL group than in the LSIL/CIN1 group. Additionally or alternatively, biopsy-negative LSILs may represent currently regressing lesions. This may be supported partially by the higher percentages of women in the biopsy-negative LSIL group who did not have cervical cancer risk factors, such as detection of hrHPVs, smoking, higher sexual activity and infections with *Chlamydia trachomatis* [13–16]. Several studies have reported that LSIL is more likely to regress to normal cytology among hrHPV-negative women or women who never smoked [5, 6, 17]. Interestingly, the 12-month regression rate of biopsy-negative LSIL was high, even among hrHPV-positive women. Low-grade lesions currently regressing to normal cytology may be difficult to confirm by colposcopy-guided biopsies because of the small lesion size, lower-grade colposcopic impression and/or weak pathologic findings.

Data on the natural course of biopsy-negative LSILs are limited. Pretorius et al. [18] reported that the subsequent risk of CIN3+ among women with histologically unconfirmed atypical squamous cells of undetermined significance (ASC-US) or LSIL cytology was low (1.8%). This

result was consistent with our observation; however, it was based on retrospective analyses of previous data including ASC-US cytology. In the ALTS (ASCUS-LSIL Triage Study) report [2], the risk of CIN3+ diagnosed within 2 years after unconfirmed LSIL was higher compared with the present study (6.1 vs. 0%). The difference between our results and the ALTS data may be explained by the difference in study design between the two studies. In the ALTS study, all women had an exit colposcopy and biopsy at 2 years after the semiannual follow-up by repeated cytology. Although our study subjects received both cytologic and colposcopic examinations at each visit at 3- to 4-month intervals, we did not routinely perform a colposcopic biopsy 2 years later. This may have resulted in an underestimation of the 2-year risk for CIN3+ in our study. Additionally, the sensitivity of the enrollment colposcopy may have affected the results from these two prospective studies. Recent studies have showed that initial colposcopy-directed biopsy are not as sensitive as we had previously assumed [19]. Thus, at least two directed biopsies, random biopsy or endocervical curettage are recommend to increase the sensitivity of the initial colposcopy [20–22]. In the ALTS study, 77.6% of women had null or only one biopsy at enrollment colposcopy [20]. By contrast, two (or more) biopsies were taken at entry in our study subjects. The number of biopsies may have increased the risk of misclassification errors of cervical lesions at enrollment. Although central pathologic review systems were employed in both studies, the limitation of histopathologic diagnosis (i.e., poor reproducibility in CIN grading) may also have affected disease classification at enrollment and during follow-up [7, 8, 23].

The current US guidelines advise that women with LSIL cytology and a histologic diagnosis of CIN1 or less should be followed with repeated cytology at 6 and 12 months or, alternatively, hrHPV testing at 12 months [4]. Our data also confirmed that these management strategies are sufficiently safe. A previous study reported that there was no significant difference in the subsequent risk of CIN2/3 between women with no disease documented by initial colposcopy-directed biopsy and women with histologically confirmed CIN1 [24]. However, the study was based on retrospective analyses, which was limited by the small sample size (negative biopsy $n = 43$; CIN1 $n = 30$) and included women with various cytologic abnormality profiles. In the present study, the risk of CIN3+ diagnosed within the following 2 years and the likelihood of LSIL regression were obviously different between women with biopsy-negative LSILs and women with LSIL/CIN1. The 2-year follow-up in ALTS of women with CIN1 or less has indicated that the subsequent risk of CIN2 or higher varies little with respect to the findings at the initial colposcopy [2]. However, when the analysis was confined to the risk of CIN3 or higher among women with LSILs, there was a marginal tendency for a higher risk of subsequent CIN3 that was associated with CIN1 compared with <CIN1 (10.5 vs. 6.1%). Based on these observations, the follow-up strategy for women with biopsy-negative LSILs may be better differentiated from that for women with LSIL/CIN1 results in terms of quality-of-life and cost. Our data suggest that follow-up by repeated cytology at 12 months may be appropriate for women with biopsy-negative LSIL when two or more colposcopy-directed biopsies are taken at the initial colposcopy.

In conclusion, the risk of CIN3+ diagnosed within 2 years was low in women with biopsy-negative LSILs; furthermore, approximately 70% showed cytologic regression within 12 months, regardless of HPV testing results. Our data suggest that biopsy-negative LSILs may represent false-positive cytology or currently regressing lesions rather than lesions missed by colposcopy. However, the sample size of the present study was small; thus, to confirm our results, further prospective studies with larger sample sizes will be needed.

Acknowledgments The authors thank Dr. Tadahito Kanda (Center for Pathogen Genomics, National Institute of Infectious Diseases, Tokyo, Japan) for his comments on the study design and the manuscript; Mr. Masafumi Tsuzuku (Department of Cytopathology, Cancer Institute Hospital, Japanese Foundation of Cancer Research, Japan) for his cytologic review; many other researchers who facilitated this study; and all the women who participated in the study. This work was supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan (grant # 12218102).

Conflict of interest We declare that we have no conflict of interest relevant to this article. The supporting organization played no role in the design and implementation of the study; the collection, management, analysis, and interpretation of the data; and the preparation, review, or approval of the manuscript.

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ORIGINAL ARTICLE

A Possible Coagulation-Independent Mechanism for Pregnancy Loss Involving $\beta_2$glycoprotein 1-Dependent Antiphospholipid Antibodies and CD1d

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Keywords
Antibody cross-linking, CD1d, phosphatidylserine, recurrent pregnancy loss, $\beta_2$glycoprotein1 (β2GP1) antibody

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Submitted March 8, 2011;
accepted April 28, 2011.

Citation
Iwasawa Y, Kawana K, Fujii T, Schust DJ, Nagamatsu T, Kawana Y, Sayama S, Miura S, Matsumoto J, Adachi K, Hyodo H, Yamashita T, Kozuma S, Taketani Y. A possible coagulation-independent mechanism for pregnancy loss involving $\beta_2$glycoprotein 1-dependent antiphospholipid antibodies and CD1d. Am J Reprod Immunol 2012; 67: 54–65

doi:10.1111/j.1600-0897.2011.01028.x

Problem

$\beta_2$glycoprotein1 (β_2GP1)-dependent antiphospholipid antibodies (aPL) increase the risk for recurrent pregnancy loss. We address whether anti-β_2GP1 antibodies can interact with phosphatidylserine (PS)-bearing CD1d on trophoblast cells and induce local inflammation.

Methods

CD1d-bearing choriocarcinoma cells were used in flow cytometry and immunoprecipitation experiments. CD1d-mediated cytokine induction was assessed using antibody cross-linking. Cytokine production during co-culture of decidual lymphocytes with CD1d-bearing cells was also examined.

Results

Trophoblast surface-expressed CD1d forms a complex with PS-bound β_2GP1. Anti-β_2GP1 mAb cross-linking causes IL12p70 release from CD1d-bearing cells. IL12p70 release from CD1d-bearing trophoblast cells was also induced during co-culture with human decidual lymphocytes. The addition of anti-β2GP1 mAb to co-cultures resulted in a three-fold increase in IL12p70 secretion. IFNγ secretion from decidual lymphocytes was also induced during co-culture with anti-β2GP1 mAbs.

Conclusions

β_2GP1-dependent IL12 release from CD1d-bearing trophoblast in the presence of aPL may link the antiphospholipid syndrome to pregnancy loss via an inflammatory mechanism.

Introduction

Antiphospholipid syndrome (APS) is characterized by the production of autoantibodies against negatively charged membrane phospholipid-dependent antigens, including cardiolipin and phosphatidylserine. APS is clinically associated with thrombocytopenia, thrombosis, and pregnancy loss.[1,2] The major target antigen for antiphospholipid antibodies (aPL) has recently been identified as β_2GP1. β_2GP1 molecule is present in the peripheral blood and can act as an inhibitor of the intrinsic coagulation cascade,[3] platelet aggregation, and the prothrombinase activity of activated platelets *in vitro*.[4,5] The role of anti-β2GP1 antibodies in APS-related pregnancy loss may involve interference with the activity of β2GP1 bound to phospholipids on

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activated platelets and the induction of coagulation in the placenta.[6,7] This would suggest that anti-β2GP1 antibody-mediated pregnancy loss occurs after the establishment of blood flow through the placental vasculature (i.e. 9–10 weeks of gestation) and would exclude those anti-β2GP1 antibody-mediated losses known to occur during the first trimester. Examination of first-trimester decidua and trophoblast tissues from pregnancies complicated by the APS, however, has revealed little evidence of specific thrombotic placental pathology.[8] Pure thrombotic events cannot account for all the histopathologic findings in placentae from women with APS.[9]

CD1d is an MHC I-like molecule that presents self- or microbe-derived glycolipid rather than peptide antigens.[10] Its immune effectors are typically natural killer T (NKT) cells.[10] CD1d presents lipid antigens including bacterial and self-lipid. Phosphatidylserine (PS), phosphatidylethanolamin (PE) and phosphatidylinositol (PI) have been known to be presented by CD1d.[11] In humans, a specific subset of NKT cells expresses an invariant Vα24Jα18/Vβ11 T-cell receptor (iTCR) and can recognize CD1d on the surface of antigen-presenting cells (APCs) through this receptor. The activation of invariant NKT (iNKT) cells is antigen dependent, but the antigen itself can be derived from an invading microbe or possibly the host itself.[12] Recognition of CD1d by iNKT cells causes rapid release of IL4 and IFN-γ from the iNKT cell and thereby modulates the Th1/Th2 polarization of adaptive immune cells.[10] The function of CD1d can be addressed experimentally using monoclonal antibody (mAb) cross-linking of cell surface-expressed CD1d. CD1d cross-linking using an anti-CD1d mAb (51.1) induces tyrosine phosphorylation in the CD1d cytoplasmic tail, intracellular signaling through NF-κB and autocrine cytokine release from CD1d-bearing cells.[13,14] As CD1a does not have a tyrosine in its cytoplasmic tail, this cascade does not occur upon cross-linking of CD1a or chimeric molecules that include the CD1a cytoplasmic tail (e.g. CD1d/a, a chimera composed of the extracellular and transmembrane domains of CD1d and the cytoplamic tail of CD1a).[13]

Normal placental extravillous trophoblast cells (EVT) express the MHC class I-like molecule, CD1d, when analyzed by immunohistochemistry.[15] We have reported that CD1d is expressed on the surface of early extravillous trophoblast cells using transient primary cultures of cells isolated from normal placentae during early pregnancy.[16] We have also demonstrated that CD1d expression is regulated *in vivo* in a trophoblast differentiation-dependent manner. CD1d is strongly expressed in EVT located proximally in trophoblast cell columns.[17] Here, the proximity of EVT and iNKT cells would allow interaction of CD1d and its ligand during early placental formation, a process that requires a local pro-inflammatory milieu to promote invasion of EVT into the maternal decidua.[17] CD1d expression in EVT decreases in the distal trophoblast cell columns that invade into the decidua and differentiate into interstitial or endovascular trophoblast cells.[17] These distal EVT cells are closely opposed to a great number of decidual stromal cells and infiltrating decidual immune cells (including decidual iNKT cells). Both NKT cells in the decidua and the peripheral blood had an ability to rapidly produce cytokine associated with Th1 and Th2.[18] It has reported that the percentages of IL4 and IFNγ producing NKT cells were significantly increased in the decidua compared with the peripheral blood in pregnancy.[18] As massive activation of iNKT cells induces pregnancy loss [19,20] the activation of decidual iNKT cells must be tightly regulated. Decreased CD1d expression in distal cell columns may control global activation of iNKT cells. More detailed descriptions of human placental CD1d regulation and expression patterns have been difficult to obtain using stable culture system.

We hypothesized that some APS-associated pregnancy loss may be mediated by anti-β_2GP1 antibody-dependent inflammation at the maternal–fetal interface. We further implicate trophoblast-expressed CD1d molecules as integral to induction of this inflammation. In this work, we demonstrate that β_2GP1-dependent aPL promote IL12 release from CD1d-bearing extravillous trophoblast cell lines, and subsequent IFNγ production by decidual lymphocytes. This, in turn, could cause further activation of inflammatory cells, damage to trophoblast cells, and pregnancy loss.

Materials and methods

Cell Lines

Although we have reported a transient primary culture system for human trophoblast cells isolated from normal placenta during early pregnancy,[21] these cells are difficult to maintain in culture. We therefore used the well-described human trophoblast cell line, Jeg3, for the present studies. While Jeg3 cells are often used *in vitro* to represent extravillous

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cytotrophoblast cells, they do not endogenously express CD1d, which is expressed only in proximal cell column EVT *in vivo*.[16] We therefore transduced CD1 genes into Jeg3 cells using a retroviral vector, pSR-neo, expressing CD1d or a CD1d/a chimeric molecule (kind gifts from Dr. R. S. Blumberg, Harvard Medical School, Boston, MA, USA).[13] Transduced cells were cultured in RPMI1640 (Invitrogen Corporations, Carlsbad, CA, USA) media supplemented with 10% FBS (Invitrogen Corporations) and 500 μg/mL of geneticin/neomycin (Invitrogen Corporations). Neomycin-resistant stable cell lines were established and named Jeg3/CD1d or Jeg3/CD1d/a cells. The chimeric molecule expressed in Jeg3/CD1d/a cells consisted of the extracellular and transmembrane components of CD1d fused to the cytoplasmic tail of CD1a.

Isolation and Culture of Human Decidual Lymphocytes

Human decidual iNKT cells were purified from human decidual tissue isolated after elective termination of uncomplicated first trimester pregnancies under the approval of the Ethical Committee of the Medical Faculty, University of Tokyo. Purified decidual iNKT cells were cultured as previously described.[22] Briefly, minced decidual tissues were digested with 10% collagenase (20 μL) and 20 U/mL DNase type1 (Invitrogen Corporations) in 10 mL RPMI 1640 containing 10%FBS and passed through mesh (100 μm pore size; Invitrogen Corporations). Cells were resuspended in PBS, layered over 5 mL of Ficoll (Sigma-Aldrich Inc., St. Louis, MO, USA) and centrifuged at $800 \times g$ for 20 min at room temperature. Cells at the interface between the PBS and Ficoll layers were collected, resuspended in RPMI1640 containing 10% FBS and 100 ng/mL of αGalCer and cultured at 37°C in 5% CO_2. An aliquot of the isolated lymphocytes was incubated with anti-Vα24-RPE or anti-Vβ11-FITC Abs (1 μg/mL) and analyzed by flow cytometry to confirm the presence of Vα24Vβ11 iNKT cells (data not shown).

In co-culture experiments, 10^5 αGalCer-stimulated decidual lymphocytes were incubated with 10^5 Jeg, Jeg/CD1d, or Jeg/CD1d/a cells at 37°C in 5% CO_2 for the times indicated. For experiments requiring exposure to anti-β2GP1 antibodies, anti-β2GP1 antibody or isotype-control antibodies were added to culture supernatants at a concentration of 10 μg/mL.

Flow Cytometry

Jeg3 cells were detached from culture plates with 0.05% EDTA in PBS and incubated with anti-CD1d (NOR3.2 Abcam Inc., Cambridge, MA, USA), anti-$β_2$GP1 (CHEMICON International, Temecula, CA, USA) or anti-PS (upstate (Millipore), Billerica, MA, USA) mAbs (1 μg/mL) in PBS for 30 min. For indirect staining experiments, cells were incubated with RPE anti-mouse IgG (Dako Cytomation, Glostrup, Denmark) for 30 min. Controls were exposed to an isotype-matched irrelevant mAb (1 μg/mL; Dako Cytomation). After washing, cells were subjected to flow cytometric analysis for RPE.

Immunoprecipitation and Western Immunoblotting

Cells were cultured in 10-cm plates in RPMI and used at near confluence. Equivalent aliquots of cell lysates were incubated overnight at 4°C with 10 μg/mL of anti-CD1d mAb or anti-AnnexinV Ab (BioVision, Mountain View, CA, USA) and 60 μL of Protein-G-Sepharose (GE Healthcare Bio-science, Piscataway, NJ, USA). As PS cannot be detected by Western blotting, these immunoprecipitants were immunoblotted with a mAb to Annexin V, a protein known to bind tightly to PS.[23] Precipitated proteins were separated across 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. $β_2$GP1 levels were determined by Western immunoblotting (ECL advance Western blotting detection kit; GE Healthcare Bio-science, Piscataway, NJ, USA). The expression of $β_2$GP1 was detected using an anti-$β_2$GP1 mAb labeled with HRP (Peroxidase Labeling Kit; Roche Diagnostics, Lewes, UK).

CD1d Cross-Linking

Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells were cultured in 12-well plates in RPMI and used at 80% confluence. Monolayer cells were exposed to ten [10] μg/ml of anti-CD1d (51.1; eBioscience, San Diego, CA, USA), anti-$β_2$GP1 (CHEMICON International) or isotype control (DakoCytomation) mAbs and incubated for 1 hr at 37°C. This anti-$β_2$GP1 antibody is non-functional and binds to two molecules of $β_2$GP1. Cells were washed with PBS and exposed to 10 μg/mL goat anti-mouse Ig antibody (CHEMICON International) as a cross-linker for 30 min at 37°C. Cells used in the anti-$β_2$GP1 mAb experiments were

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not exposed to a secondary antibody. Cells were then washed three times and incubated in the serum-free growth media without antibiotics for periods of 0, 18, or 24 hr prior to use for RT-PCR or ELISA.

Quantitative RT-PCR

Quantitative IL12 p40 RT-PCR was performed in cultured Jeg3 cells after cross-linking. Total RNA was isolated (RNeasy; Qiagen Inc., Valencia, CA, USA) and 2 μg of total RNA were subjected to reverse transcription (ReverTraAce; TOYOBO, Tsuruga, Japan) according to the manufacturer's instructions. A volume of 0.2–2 μL of each RT-reaction was used for quantitative PCR (Light Cycler system; Roche Diagnostics, Lewes, UK) using the following primer pairs: IL12 p40-forward 5′-GGTCACACTGGA CCAAAGGGACTATG-3′, -reverse 5′- ATTCTGCTGCC GTGCTTCCAAC-3′[24]; and β-actin-forward 5′- GA-AATCGTGCGTGACATTAAGG-3′, -reverse 5′- TCAG GCAGCTCGTAGCTTCT C-3′.[25] β-actin mRNA levels were quantified in each sample as an internal control for normalization. RT-quantitative PCR experiments were repeated at least three times.

ELISA for IL12 and IFNγ

Culture media was collected at 0, 18, and 24 hr after cross-linking ($n = 4$) and levels of secreted IL12 p70 or IFNγ were quantified using solid phase sandwich ELISAs (R&D Systems). A standard curve was produced using absorbance measurements at 450 nm for standard samples. Each unknown sample was similarly measured and plotted using this standard curve. IL12 and IFNγ secretion levels in the presence of the anti-β2GP1 antibody were normalized by those in the absence of the antibody and indicated as ratios.

Statistical Analysis

Quantitative PCR and ELISA data are presented as means ± standard deviations. All experiments were performed independently three times. mRNA or cytokine secretion levels were compared between cell lines and among time points using Mann–Whitney analyses. A P-value of <0.05 was considered significant.

Results

Cell-Surface Expression of PS and β_2GP1 Bound to CD1d

CD1d is an MHC-like glycoprotein that presents lipid antigens derived from pathogens and from self, the latter including phosphatidylserine (PS).[11] Phosphatidylserine can associate with β2GP1 at the surface of trophoblast cells.[26,27] The carrier involved in intracellular trafficking and transport of PS-β2GP1 complexes to the cell surface has not been identified. We hypothesized that the PS-β2GP1 complex is presented by CD1d in trophoblast cells. To test our hypothesis, we developed a trophoblast cell model that remained undifferentiated with prolonged *in vitro* culture, but stably expressed CD1d at the cell surface. Jeg3 cells are a commonly used model for extravillous trophoblast cells, but these cells do not endogenously express CD1d (Fig. 1a, green line). We transduced CD1d into Jeg3 cells using a retroviral expression system to establish the stable Jeg3/CD1d cell line with strong cell surface expression of CD1d (Fig. 1b, green line). Jeg3 and Jeg3/CD1d cells were triple-stained for PS, β2GP1, and CD1d and analyzed by flow cytometry (Fig. 1). Both PS (red lines) and β2GP1 (blue lines) were expressed on the cell surface of Jeg3/CD1d with signal peaks that overlapped that of CD1d (green lines). Neither PS nor β2GP1 was expressed on the cell surface of Jeg3 that lacked CD1d.

Using similar methodologies, we generated a cell line expressing a chimeric CD1 molecule comprised of the extracellular domains of CD1d and intracellular domain of CD1a (CD1d/a). As CD1a lacks a tyrosine in its cytoplasmic tail, downstream tyrosine-based signaling does not occur upon ligand binding to chimeric surface CD1d/a molecules.[13] The resultant cell line, called Jeg3/CD1d/a cells, can be used as a dominant-negative control in CD1d cross-linking experiments. As with Jeg3/CD1d cells, PS and β2GP1 were expressed on the surface of Jeg3/CD1d/a cells (Fig. 1c). Flow cytometry patterns for PS and β2GP1 are nearly identical in Jeg3/CD1d and Jeg3/CD1d/a cells, suggesting that PS and β2GP1 may form a complex at the cell surface and indicating that cell surface expression of PS and β2GP1 depends on the presence of the extracellular domains of CD1d.

To address biochemical interactions between CD1d and the PS-β2GP1 complex, an anti-CD1d mAb was

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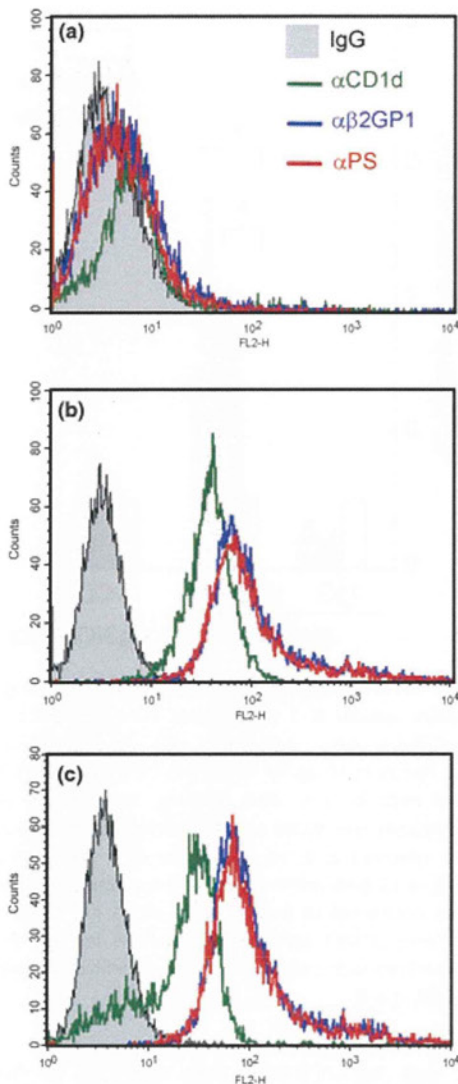

Fig. 1 Cell-surface expression of PS and β_2GP1 is CD1d-dependent. Jeg3 (a), Jeg3/CD1d (b) and Jeg3/CD1d/a (c) cells were triple stained with the anti-CD1d mAb (green), the anti-PS Ab (red) and anti-β2GP1 mAb (blue) and analyzed using flow cytometry. Background staining with isotype-matched control antibody is also shown (black). Histograms are representative of at least four separate experiments. (Blue lines (β2GP1) merged red lines (PS) in each panel of Fig. 1 because of the co-localization of the two molecules).

used to co-precipitate CD1d and its associated molecules from total cell lysates of Jeg3 and Jeg3/CD1d cells. These immunoprecipitants were then immunoblotted to detect β2GP1 (Fig. 2a). A 42-KDa band representing β2GP1 was present only in the Jeg3/CD1d cells, although immunoglobulin heavy chains were detected in all cells. Interaction between Cd1d and PS was similarly verified using co-immuno-

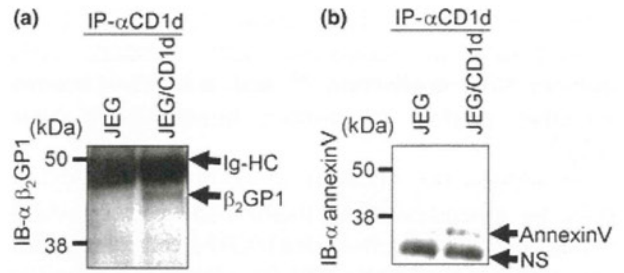

Fig. 2 The PS-β_2GP1 complex binds to CD1d. An anti-CD1d mAb was used to immunoprecipitate CD1d from Jeg3 or Jeg3/CD1d total cell lysates. (a) Immunoprecipitants were immunoblotted with an HRP-labeled anti-β_2GP1 mAb to detect β2GP1. An extra-band at 42 KDa, representing β_2GP1, was observed only in the Jeg3/CD1d lane although bands representing immunoglobulin heavy chains were noted in both lanes. (b) The immunoprecipitants were immunoblotted with an anti-Annexin V mAb to detect Annexin V, a PS-binding protein. An extra-band at 35 KDa, representing annexin V, was observed only in the Jeg3/CD1d lane although bands representing immunoglobulin light chains were noted in both lanes.

precipitation and Western blotting. Again, primary immunoprecipitations used an anti-CD1d mAb. As PS cannot be detected by Western blotting, these immunoprecipitants were immunoblotted with a mAb to Annexin V, a protein known to bind tightly to PS[23] (Fig. 2b). Although we could not detect PS directly, these biochemical data suggested the hypothesis that the PS-β2GP1 complex is expressed on the trophoblast cell surface and is bound to CD1d.

Ligation of Cell Surface CD1d Promotes IL12 Release from Trophoblast Cells

Ligation of surface-expressed CD1d promotes rapid but transient cytokine secretion from CD1d-bearing cells.[13,28,29] Such ligation can also occur upon interaction with iTCR-expressing iNKT cells and via antibody cross-linking of CD1d.[28] The cytoplasmic tail of CD1d, but not CD1a, bears a target domain for potential tyrosine kinase activity characterized by the tyrosine endocytic sorting motif (YXXZ).[13] CD1d ligation induces tyrosine phosphorylation in its cytoplasmic tail, subsequent intracellular signaling, and autocrine cytokine release from CD1d-bearing cells.[14,28] The 51.1 anti-CD1d mAb is often used for CD1d cross-linking and its use creates an *in vitro* model for CD1d ligation.[13,14] We have reported that cross-linking of CD1d using 51.1, when combined with secondary anti-Ig antibodies, promotes IL12 and IL15 secretion from reproductive tract epithelial

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cells.[29] Other groups have shown that similar CD1d cross-linking in monocytes and dendritic cells induces IL12 production [28] and IL12 is a known secretion product of normal human trophoblast cells.[30]

To address the function of CD1d in trophoblast cells, we examined CD1d ligation-induced autocrine cytokine production from Jeg3/CD1d cells. Jeg3 cells expressing the chimeric CD1d/a (Jeg3/CD1d/a cells) were used as dominant-negative control, as chimeric CD1d/a (extracellular CD1d and cytoplasmic CD1a) bears no target motif for phosphorylation. Both cell lines were first exposed to an anti-CD1d 51.1 mAb or to an isotype-control mAb. This was followed by exposure to a secondary anti-mouse IgG antibody cross-linker. The cells were then examined for IL-12 transcription (Fig. 3) using RT-PCR. Transcription of IL12 (p40) increased relatively rapidly (18 hr after cross-linking) in Jeg3/CD1d cells exposed to the CD1d-specific 51.1 mAb but did not increase in those exposed to isotype-control antibody. Although Jeg3/CD1d/a cells express cell-surface CD1d (shown in Fig. 1c), IL12 production was not induced in these cells upon exposure to anti-CD1d mAb or isotype control antibodies, indicating that IL12 production is mediated through pathways involving the cytoplasmic tail of CD1d.

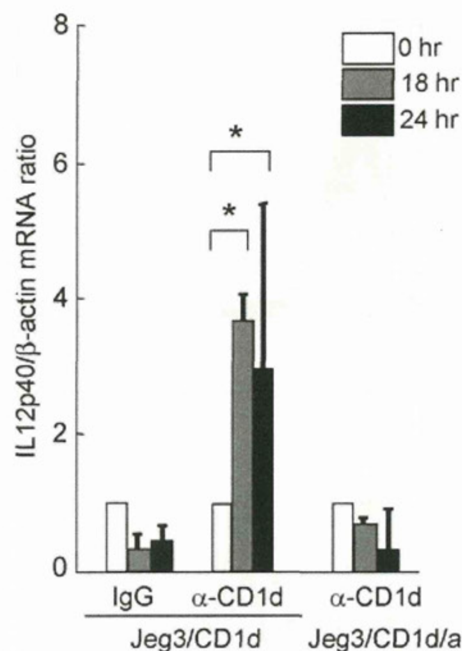

Fig. 3 CD1d cross-linking using anti-CD1d primary and anti-Ig secondary antibodies induces IL12 transcription from Jeg3/CD1d cells but not Jeg3/CD1d/a cells. Jeg3/CD1d cells or Jeg3/CD1d/a were exposed in culture to 10 μg/mL of the anti-CD1d mAb (51.1) or an isotype-control mAb for 1 hr. After washing, 10 μg/mL of goat anti-mouse Ig antibody was added as a cross-linker for 30 min to all cells. Cells were harvested at 0, 18, and 24 hrs after secondary antibody cross-linking. IL-12 (p40) mRNA levels were analyzed using quantitative RT-PCR and normalized to β-actin. Mean mRNA levels and standard deviations were plotted against time. Asterisks indicate those time point comparisons with statistical significance within a cell-line exposure ($P < 0.05$; $n = 4$).

An anti-β2GP1 Monoclonal Antibody Induces Autocrine IL12 Production from Trophoblast Cells by CD1d Ligation in the Absence of a Secondary Cross-Linker

Existing *in vitro* models for antibody cross-linking of CD1d requires use of primary and secondary antibodies.[13,28,29] Several investigators have demonstrated that one anti-β2GP1 antibody binds two cell-surface β2GP1 molecules.[31] In light of our finding that β2GP1 appears to be bound to cell-surface CD1d molecules via PS, we hypothesized that a single anti-β2GP1 antibody might ligate two CD1d molecules via their bound PS-β2GP1 complexes. This CD1d ligation would not require use of secondary antibodies and thereby could commonly occur *in vivo*. To address this hypothesis, Jeg3/CD1d and Jeg3/CD1d/a cells were exposed to anti-β2GP1 mAbs in the absence of secondary cross-linking antibodies. Post-exposure RT-PCR revealed that transcription of IL12 (p40) increased in Jeg3/CD1d cells 18 and 24 hr after exposure to anti-β2GP1 mAb alone (Fig. 4). Jeg3/CD1d cells exposed to isotype control and Jeg3/CD1d/a cells exposed to the anti-β2GP1 mAb exhibited no change in IL12 transcription when analyzed up to 24 hr post-exposure. Notably, the increase in IL12 transcription after anti-β2GP1 mAb exposure was greater than that seen after combined anti-CD1d and secondary antibody exposure (Fig. 3).

Autocrine Secretion of IL12 from CD1d-Bearing Trophoblast Cells upon CD1d Ligation

To examine autocrine IL12 production from CD1d-bearing JEG3 cells after CD1d ligation, protein secretion into the culture media was assessed by ELISA (Fig. 5). Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells were exposed to an anti-CD1d mAb (51.1) then to secondary antibody cross-linker as described previously. In separated cultures, these same cells were also

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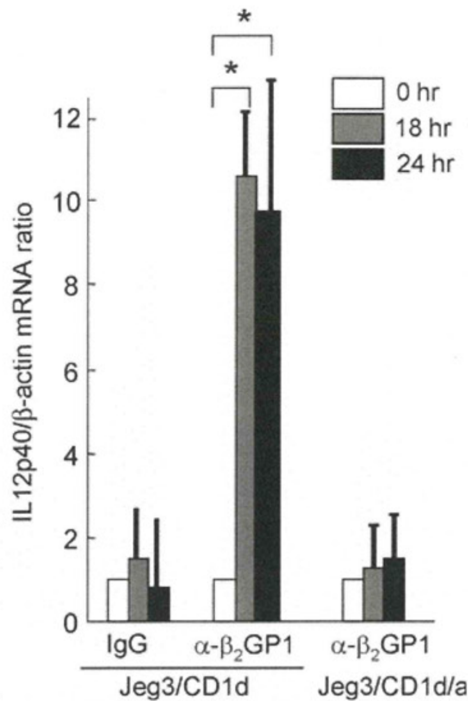

Fig. 4 Ligation of CD1d/PS/β_2GP1 complexes on Jeg3/CD1d cells using only anti-β_2GP1 mAbs increases IL12 transcription. Anti-β2GP1 and isotype-matched control mAbs were used as cross-linking antibodies in the absence of a secondary cross-linking reagent. Cells were harvested at 0, 18, and 24 hrs after cross-linking. IL-12 (p40) mRNA levels were analyzed using quantitative RT-PCR and normalized to β-actin. Mean mRNA levels and standard deviations were plotted against time. Asterisks indicate those time point comparisons with statistical significance within a cell-line exposure ($P < 0.05$; $n = 4$).

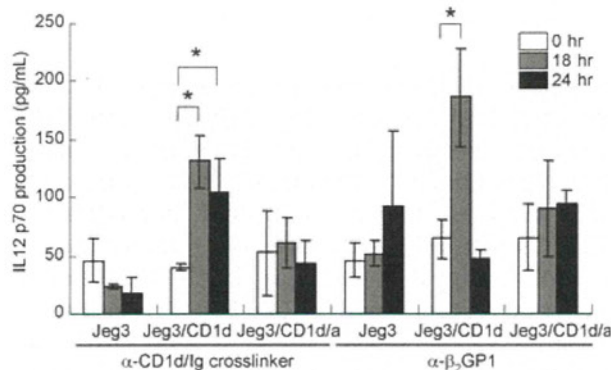

Fig. 5 Ligation of CD1d on Jeg3/CD1d cells using either anti-CD1d or anti-β_2GP1 mAbs increases IL12 secretion from Jeg3/CD1d cells. After cross-linking by anti-CD1d or anti-β_2GP1 mAb as shown in Fig. 4, cells were incubated in serum-free growth media without antibiotics for periods of 0–24 hrs. Autocrine IL12 secretion from trophoblast cell lines at each time point was assessed by ELISA for IL-12 (p70). Mean values with standard deviations are presented. Asterisks indicate those comparisons (before versus after cross-linking and among time points) with statistical significance ($P < 0.05$; $n = 4$).

exposed to an anti-β2GP1 mAb in the absence of a cross-linker. IL12 secretion increased in Jeg3/CD1d cells in both conditions, but neither exposure affected IL12 secretion in Jeg3/CD1d/a cells. IL12 secretion peaked at 18 hr after both anti-CD1d/secondary antibody and anti-β2GP1 cross-linking in Jeg3/Cd1d cells. Peak IL12 secretion levels were approximately three-fold higher than baseline levels after anti-β2GP1 mAbs cross-linking. The increase in IL12 secretion was higher after exposure to anti-β2GP1 mAbs alone when compared with combined anti-CD1d mAb/secondary antibody exposure, although the difference was of marginal significance ($P = 0.055$). These results again support the hypothesis that the induction of IL12 production requires CD1d-mediated intracellular signaling.

IL12 Production from CD1d-Bearing Trophoblast Cells upon Co-Culture with Normal Human Decidual Lymphocytes

It is reported that the percentages of NKT cells were significantly increased in the decidua compared with the peripheral blood.[18] Boyson et al.[19] has reported that CD1d-restricted $V\alpha24^+V\beta11^+$ iNKT cells comprise 0.48% of $CD3^+$ lymphocytes isolated from human decidual tissues at 7–9 gestational weeks. This is much higher than the 0.04% frequency in peripheral blood. To better mimic occurrences at the human maternal–fetal interface in normal pregnancies, we developed an *in vitro* model that exposed CD1d-bearing trophoblast-derived cells to decidual lymphocytes from normal human pregnancies at 7–9 weeks of gestation. Decidual lymphocytes were stimulated with αGalCer, a specific ligand for iTCR, to increase the proportion of $V\alpha24^+$ $V\beta11^+$ iNKT cells prior to co-culture with Jeg3 or Jeg3/CD1d cells. The proportion of the iNKT cells increased from 0.4–0.5 to 7–8% of decidual lymphocytes after αGalCer stimulation, but did not differ by the gestational age of the pregnancy from which the lymphocytes were isolated (data not shown). Jeg3 or Jeg3/CD1d cells were then cultured with or without decidual lymphocytes derived from pregnancies at either 7 or 9 gestational weeks; lymphocytes from a single pregnancy were used for all exposures within a single experiment. Decidual lymphocytes from the same pregnancy were cultured alone as an internal control (Figs 6–8). Secretion of IL12 increased 18 hrs after exposure of Jeg3/CD1d cells to decidual lymphocytes in co-culture, while neither co-culture of decidual

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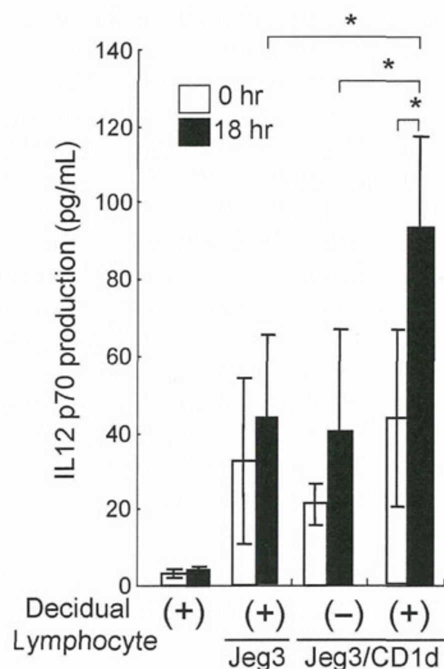

Fig. 6 IL12 secretion from Jeg3/CD1d cells is induced by the presence of decidual lymphocytes that include CD1d-restricted iNKT cells. Decidual lymphocytes were isolated from normal human decidual tissues collected at 7–9 gestational weeks. 10^5 decidual lymphocytes were cultured with Jeg3 or Jeg3/CD1d cells. Jeg3/CD1d cells alone and decidual lymphocytes alone were also cultured as controls. Autocrine IL12 secretion from trophoblast cells at the 18 hrs time point was assessed by ELISA for IL-12 (p70). Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistical significance ($P < 0.05$; $n = 4$).

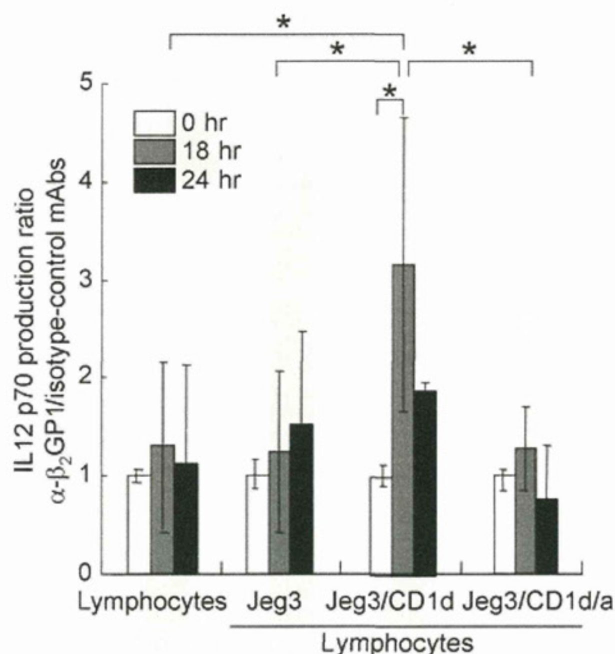

Fig. 7 Anti-β_2GP1 mAb enhances IL12 secretion during co-culture of Jeg3/CD1d cells and decidual lymphocytes. Decidual lymphocytes were co-cultured with Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells. Decidual lymphocytes were also cultured alone as an internal control. Cultured cells were exposed to anti-β_2GP1 or control mAbs for 24 hrs (10 μg/mL). IL12 p70 levels in medium collected from each culture at each time point was measured by ELISA. The ratio of IL12 production after anti-β_2GP1 and control mAbs exposure is depicted (anti-β_2GP1/control mAb). Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistically significant differences ($P < 0.05$; $n = 4$).

lymphocytes alone, Jeg3 cells and decidual lymphocytes nor of Jeg3/CD1d cells alone induced IL12 production (Fig. 6). This *in vitro* model recapitulates physiological interactions between CD1d-bearing trophoblast cells and decidual lymphocytes at the maternal–fetal interface in the absence of antibody-mediated stimulation. The increases in IL12 production in this model were less than those seen in antibody-cross-linking experiments that modeled pathological conditions.

Anti-β2GP1 mAbs Enhance Cytokine Induction by CD1d-Bearing Trophoblast in Co-Culture with Decidual Lymphocytes

Autocrine IL12 secretion from CD1d-bearing trophoblast cells was next induced by either antibody cross-linking or interaction with decidual lymphocytes, including CD1d-restricted iNKT cells. Here, we attempted to mimic the microenvironment at the maternal–fetal interface when pathologic anti-β2GP1 mAbs are present (Figs 7 and 8). Decidual lymphocytes derived from normal 7–9 week pregnancies were co-cultured alone, with Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells in the presence of anti-β2GP1 or control mAbs. IL12 secretion levels in co-culture media were first measured by ELISA to assess the response of trophoblast cells to anti-β2GP1 and expressed as a ratio of anti-β2GP1 mAb exposure: control mAb exposure to reveal specific effect of anti-β2GP1 mAbs (Fig. 7). A statistically significant, 3-fold increase in IL12 production ratios were noted 18 hrs after mAb exposure in Jeg3/CD1d cells. IL12 secretion ratios were also significantly increased in Jeg3/CD1d cells when compared with Jeg3 and Jeg3/CD1d/a cells. Cultures containing lymphocytes alone, lymphocytes with Jeg3, and lymphocytes with

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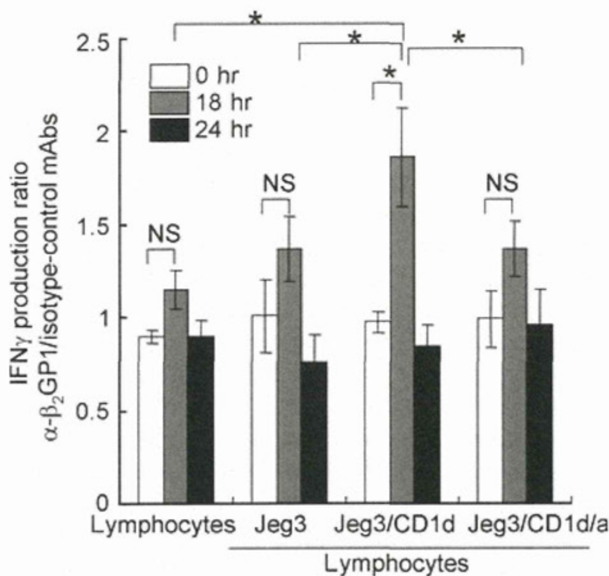

Fig. 8 Anti-β_2GP1 mAb enhances IFNγ secretion when added to co-cultures containing *Jeg3/CD1d* cells and decidual lymphocytes. IFNγ production in culture media collected for Fig. 7 was measured by ELISA and the production of IFNγ expressed as a ratio of anti-β_2GP1-exposed over control mAb-exposed specimens (anti-β_2GP1/control mAb). Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistically significant differences ($P < 0.05$; $n = 4$).

Jeg3/CD1d/a cells did not show significant alterations in IL12 production ratios. These results indicate that anti-β2GP1 mAbs enhance the CD1d-dependent IL12 production stimulated by decidual lymphocyte co-culture. They also suggest that anti-β2GP1 antibody can ligate two CD1d molecules via their attached PS-β2GP1 complexes even in the presence of background stimulation by CD1d-restricted iNKT cells.

We also used methodology similar to that which generated Fig. 7 to examine the secretion of IFNγ into co-culture media in the presence of anti-β2GP1 or control mAbs (Fig. 8). Unlike IL12, there was a trend toward increased IFNγ secretion 18 hrs after antibody exposure in all cultures. A statistically significant increase, however, was only demonstrated in co-cultures of Jeg3/CD1d cells with decidual lymphocytes. These data show that the presence of anti-β2GP1 mAbs may enhance IFNγ release in a maternal–fetal microenvironment interface in which CD1d-bearing trophoblast cells contact decidual lymphocytes.

Discussion

Despite the long-held belief that aPL-related pregnancy loss results from aberrant placental coagulation, direct examination of placental and first-trimester decidual tissues from pregnancies complicated by the antiphospholipid syndrome reveal little evidence for thrombotic placental pathology.[8] This suggests that aPL can induce pregnancy failure through alternative mechanisms. Recently, specific aPLs, called anti-β2GP1 antibodies, have been shown to directly alter trophoblast cell maturation,[8,32] giant multinuclear cell formation and invasion by EVT [24,32] and human chorionic gonadotropin secretion by syncytiotrophoblast.[11] Nakashima et al.[33] reported a possible miscarriage mechanism by which activated maternal NK cells attack the EVT and induce EVT apoptosis.

CD1d is expressed on the surface of APCs, including: dendritic cells, macrophages, B cells, and epithelial cells. Lipid antigens, derived from invading microbes or host phospholipid, are presented by CD1d to CD1d-restricted iNKT cells. In normal pregnancy, interactions between CD1d and iNKT cells activate iNKT cells and induce the release of pro-inflammatory cytokines, such as IL12, from CD1d-bearing cells.[28] IL12, in turn, induces IFNγ-producing NK, NKT, T helper and cytotoxic T-cell activity, thereby initiating a potent local inflammatory cascade. IFNγ also upregulates CD1d expression on APCs in a paracrine fashion (Fig. 9, upper panel). Several studies, including ours, have demonstrated CD1d expression in EVT isolated from early gestation human placentas.[15,19,34] Appropriate placental formation involves appropriate invasion of fetally derived EVT into the maternal decidua, a process that appears to require a localized, transient and tightly controlled pro-inflammatory reaction.[17] At the human maternal–fetal interface, CD1d-bearing EVT contact maternal iNKT cells during early placental formation. Our data demonstrate a relatively weak, but significant increase in IL12 protein levels in the media of Jeg3/CD1d and human decidual lymphocyte co-cultures in the absence of anti-β2GP1 mAbs (Fig. 6). This suggests that appropriate IL12 secretion secondary to physiologic interactions of CD1d and iNKT cells at the human maternal–fetal interface should be at relatively low levels.

Maternal antibodies are present in the spiral arteries, in the decidua and in the maternal blood bathing the placental villi. Therefore, maternal aPL have

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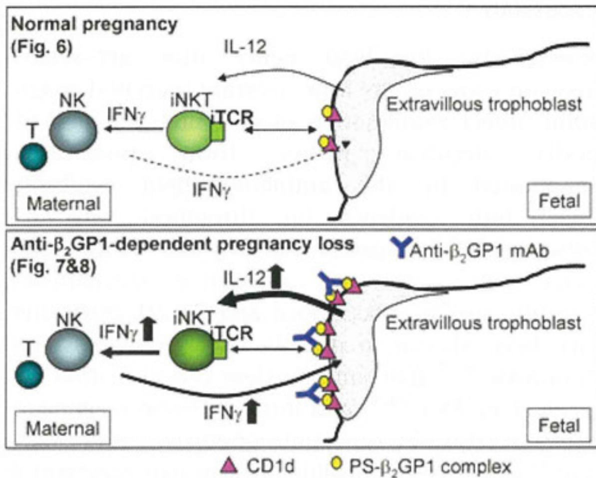

Fig. 9 Comparison of immune interactions at the human maternal–fetal interface during normal pregnancy with those proposed during anti-β_2GP1 mAb-related pregnancy loss. (Upper) In normal pregnancy, interaction of CD1d on the extravillous trophoblast cells (EVT) and maternal iNKT cells induces low level secretion of IL12 from trophoblast cells. IL12, in turn, appropriately stimulates IFNγ-producing NK, NKT, T helper and cytotoxic T cell activity, thereby initiating a potent but tightly controlled local inflammatory cascade. This enables invasion of fetally derived EVT into the maternal decidua for appropriate placental formation. (Lower) Maternal anti-β2GP1 antibodies are present in the decidua and in the maternal blood bathing placental villi. Direct interaction between anti-β2GP1 antibodies and the PS-β2GP1 complex presented by CD1 molecules ligates CD1d and induces strong IL12 production. Unchecked induction of the inflammatory cascade at the maternal interface might then result in pregnancy loss in the absence of placental coagulation.

direct access to CD1d-bearing trophoblast cells during human pregnancy. Through such interactions, maternal aPL may deter the otherwise well-localized and tightly controlled inflammatory process at the site of implantation.

In this study, we have shown that interactions between anti-β2GP1 antibodies and trophoblast CD1d molecules promote IL12 release from trophoblast cells, and IFNγ release from decidual lymphocytes via CD1d ligation. These interactions are made possible by the surface presentation of a PS-β2GP1 complex by CD1d. Although it has been previously reported that PS and β2GP1 form a complex at the cell surface,[26,27] ours is the first to demonstrate that CD1d is able to present both PS and β2GP1. We also demonstrate that CD1d ligation and downstream signaling can be initiated upon exposure to anti-β2GP1 antibodies alone without the requirement for the secondary anti-IgG antibodies necessary in standard *in vitro* CD1d cross-linking methods. As it is known that anti-β2GP1 antibodies can bind to two molecules,[31] these antibodies have the capacity to crosslink CD1d bearing β2GP1 *in vivo*. Such *in vivo* cross-linking may, in fact, be very efficient. In our *in vitro* models, ligation of the CD1d/PS/β2GP1 complex by anti-β2GP1 mAbs resulted in a much stronger transient induction of IL12 transcription than ligation of CD1d using anti-CD1d antibodies. Our co-culture data further demonstrate that the presence of decidual lymphocytes alone can stimulate downstream signaling thru trophoblast-expressed CD1d and that anti-β2GP1 mAbs can ligate the CD1d/PS/β2GP1 complex regardless of the presence of decidual lymphocytes. In fact, our data suggests that antibody-mediated ligation of CD1d in the presence of decidual lymphocytes can initiate a local inflammatory cascade via transient IFNγ release from decidual lymphocytes which are, in turn, activated by trophoblast-derived IL12. Transient cytokine release after *in vitro* CD1d ligation is known to be rapid as shown previously.[29] This might be the reason why the peak of cytokine release was observed at 18 hrs but not at 24 hrs. IL4, type 2 cytokine from iNKT cells, release was not observed in this co-culture system (data not shown).

We propose the following mechanism for anti-β2GP1 antibody-related pregnancy loss (Fig. 9, lower panel). In a mother positive for anti-β2GP1 antibodies, CD1d-bearing EVT will be exposed to these antibodies at the maternal–fetal interface during early gestation. Direct interaction between anti-β2GP1 antibodies and the PS-β2GP1 complex presented by CD1d molecules ligates CD1d and induces potent downstream IL12 production. IL12 activates maternal IFNγ-producing NK, NKT, and T cells. IFNγ derived from maternal lymphocytes upregulates CD1d expression on the surface of the EVT.[16] This overexpression of CD1d enhances anti-β2GP1 antibody-mediated cross-linking in a feed-forward fashion. Unchecked induction of the inflammatory cascade at the maternal interface could then result in pregnancy loss in the absence of placental coagulation. Further study using additional clinical materials is needed to verify this novel mechanism for aPL-related pregnancy loss.

Acknowledgements

This work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan for

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the Third-Term Comprehensive 10-Year Strategy for Cancer Control, by a cancer research grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from Kanzawa Medical Research Foundation. We are grateful to Dr. R. Blumberg of Harvard Medical School, Boston, MA, and to Dr. K. Oda of University of Tokyo for their kind gifts of the CD1d-expressing retroviral plasmid, pSRα-neo, and the retrovirus expression system, respectively.

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DOI: 10.1111/j.1471-0528.2011.03036.x
www.bjog.org

Introducing HPV vaccine and scaling up screening procedures to prevent deaths from cervical cancer in Japan: a cost-effectiveness analysis

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Accepted 15 April 2011. Published Online 28 July 2011.

Objective To assess the cost-effectiveness of universal vaccination of 11-year-old girls against human papillomavirus (HPV) infection and increased screening coverage to prevent cervical cancer in Japan where the coverage of Papanicolaou smears is very low.

Design A cost–utility analysis from a societal perspective.

Setting Japan, 2010.

Population The female Japanese population aged 11 years or older.

Methods A Markov model of the natural history of cervical cancer was constructed to compare six strategies: i.e. a screening coverage rate of 20, 50 and 80% with and without routine vaccination at age 11.

Main outcome measures Cervical cancer incidence, quality-adjusted life years (QALYs), costs and incremental cost-effectiveness ratios.

Results Expanding the coverage of Papanicolaou smears from the current level of 20–50 and 80% yields a 45.5 and 63.1% reduction in cervical cancer incidence, respectively. Impact of combined strategies increases with coverage. Coverages of 20, 50 and 80% showed a 66.1, 80.9 and 86.8% reduction in disease, respectively. The costs of strategies with vaccination are four times higher than the cost of strategies without vaccination. Vaccinating all 11-year-old girls with bivalent vaccines with a Papanicolaou smear coverage rate of 50% is likely to be the most cost-effective option among the six strategies.

Conclusions The introduction of HPV vaccination in Japan is cost-effective as in other countries. It is more cost-effective to increase the coverage of the Papanicolaou smear along with the universal administration of HPV vaccine.

Keywords Cost-effectiveness analysis, economics, human papillomavirus, vaccines.

Please cite this paper as: Yamamoto N, Mori R, Jacklin P, Osuga Y, Kawana K, Shibuya K, Taketani Y. Introducing HPV vaccine and scaling up screening procedures to prevent deaths from cervical cancer in Japan: a cost-effectiveness analysis. BJOG 2011; DOI: 10.1111/j.1471-0528.2011.03036.x.

Introduction

Cervical cancer is the fifth leading cause of female cancer death in the world.[1] The overall frequency of cervical cancer in Japan, including carcinoma *in situ*, was reported as 17 000 per year.[2] In Japan, it is the third leading cause of cancer death among women <40 years of age.[2] The age-adjusted mortality rate of cervical cancer in Japan has remained at almost the same level for the past two decades, although it has declined in the USA and UK.[2–4]

Screening with cervical cytology [i.e. Papanicolaou (Pap) smear] has been the key national strategy for early detection and treatment of cervical cancer to reduce its burden.[5] However, the coverage of Pap smear screening in Japan remains between 10 and 20%,[6] much lower than in other countries such as the UK (81%), France (54%) and the USA (>82%).[7]

Persistent human papillomavirus (HPV) infection, particularly with oncogenic types 16, 18, 52 and 58, is associated with a higher risk of incident cervical cancer precursor lesions.[8] A prophylactic vaccine to prevent infection from HPV16 and 18 to reduce the burden of cervical cancer has been developed and implemented in some countries.[9] The idea of introducing HPV vaccine in the Japanese population has evoked public debate and become a huge political

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