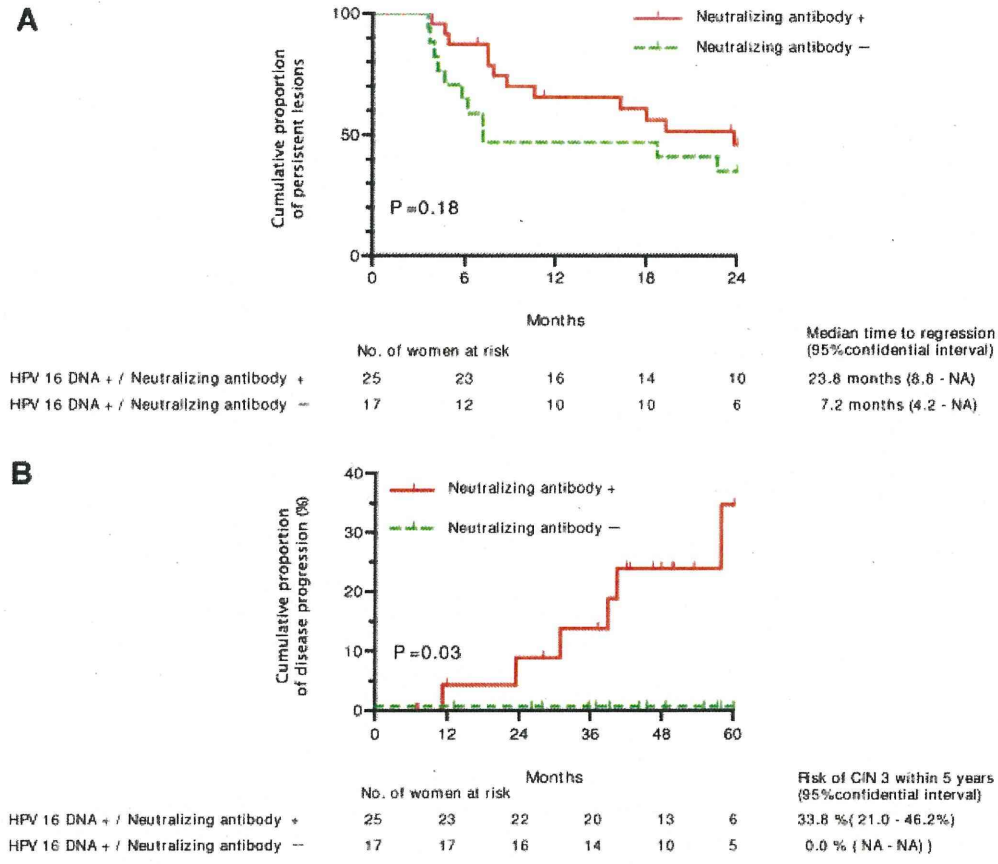

Fig. 1. Neutralizing Antibody against HPV 16 and Natural History of Low-grade Squamous Intraepithelial Lesion induced by HPV 16. Kaplan–Meier plots were used to estimate cumulative probabilities of regression (**A**) and progression (**B**) of low-grade squamous intraepithelial lesion induced by HPV 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 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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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

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

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 × ***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_2GP1 (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_2GP1 levels were determined by Western immunoblotting (ECL advance Western blotting detection kit; GE Healthcare Bio-science, Piscataway, NJ, USA). The expression of β_2GP1 was detected using an anti-β_2GP1 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_2GP1 (CHEMICON International) or isotype control (DakoCytomation) mAbs and incubated for 1 hr at 37°C. This anti-β_2GP1 antibody is non-functional and binds to two molecules of β_2GP1. 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_2GP1 mAb experiments were

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

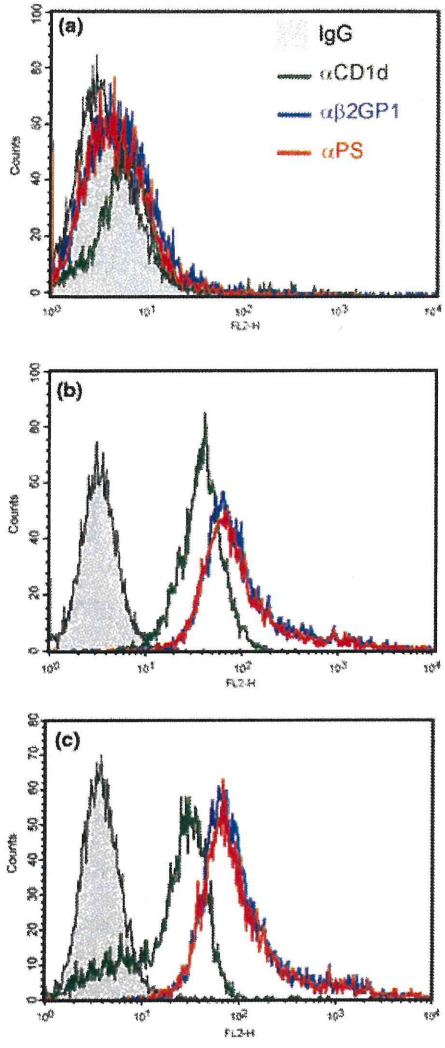

Fig. 1 Cell-surface expression of PS and β_2GP1 is CD1d-dependent. Jeg3 (a), Jeg3/CD1d (b) and Jeg3/CD1d/a (c) cells were triple stained with the anti-CD1d mAb (green), the anti-PS Ab (red) and anti-β2GP1 mAb (blue) and analyzed using flow cytometry. Background staining with isotype-matched control antibody 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-

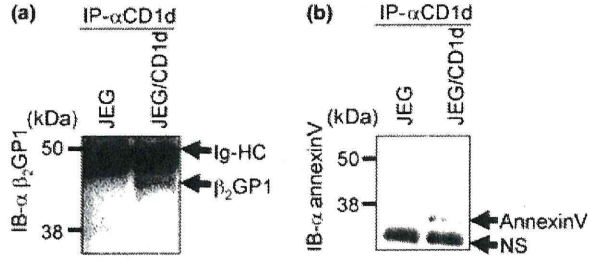

Fig. 2 The PS-β_2GP1 complex binds to CD1d. An anti-CD1d mAb was used to immunoprecipitate CD1d from Jeg3 or Jeg3/CD1d total cell lysates. (a) Immunoprecipitants were immunoblotted with an HRP-labeled anti-β_2GP1 mAb to detect β2GP1. An extra-band at 42 KDa, representing β_2GP1, was observed only in 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

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.

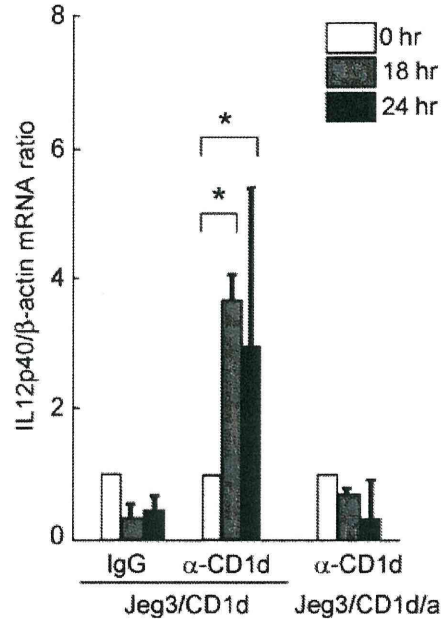

Fig. 3 CD1d cross-linking using anti-CD1d primary and anti-Ig secondary antibodies induces IL12 transcription from Jeg3/CD1d cells but not Jeg3/CD1d/a cells. Jeg3/CD1d cells or Jeg3/CD1d/a were exposed in culture to 10 μg/mL of the anti-CD1d mAb (51.1) or an isotype-control mAb for 1 hr. After washing, 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

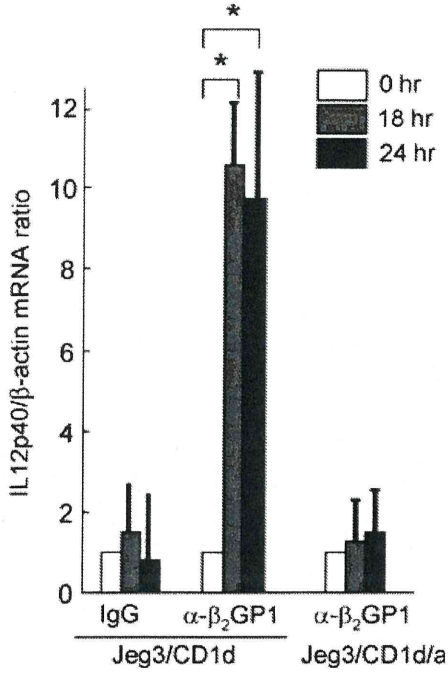

Fig. 4 Ligation of CD1d/PS/β_2GP1 complexes on Jeg3/CD1d cells using only anti-β_2GP1 mAbs increases IL12 transcription. Anti-β2GP1 and isotype-matched control mAbs were used as cross-linking antibodies in the absence of a secondary cross-linking reagent. Cells were harvested at 0, 18, and 24 hrs after 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 ($P < 0.05$; $n = 4$).

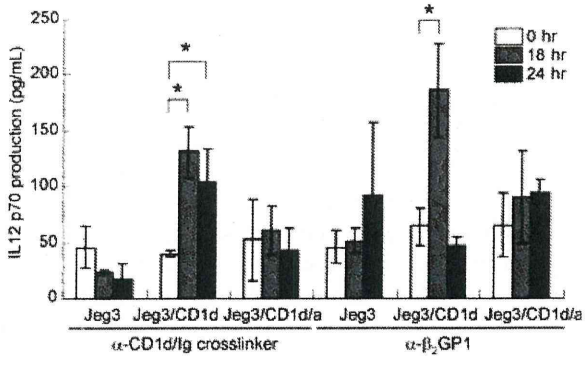

Fig. 5 Ligation of CD1d on Jeg3/CD1d cells using either anti-CD1d or anti-β_2GP1 mAbs increases IL12 secretion from Jeg3/CD1d cells. After cross-linking by anti-CD1d or anti-β_2GP1 mAb as shown in Fig. 4, cells were incubated in serum-free growth media without antibiotics for periods of 0–24 hrs. Autocrine 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

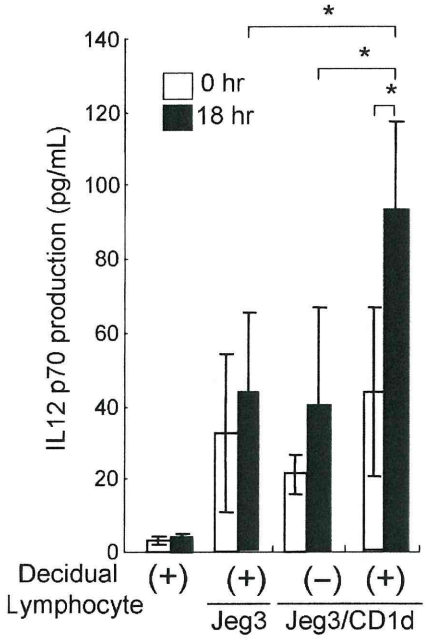

Fig. 6 IL12 secretion from Jeg3/CD1d cells is induced by the presence of decidual lymphocytes that include CD1d-restricted iNKT cells. Decidual lymphocytes were isolated from normal human decidual tissues collected at 7–9 gestational weeks. 10^5 decidual lymphocytes were cultured with Jeg3 or Jeg3/CD1d 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 ($P < 0.05$; $n = 4$).

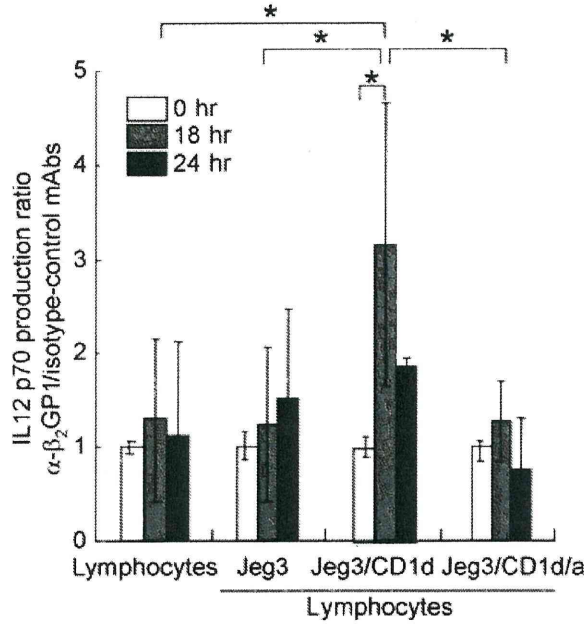

Fig. 7 Anti-β_2GP1 mAb enhances IL12 secretion during co-culture of Jeg3/CD1d cells and decidual lymphocytes. Decidual lymphocytes were co-cultured with Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells. Decidual lymphocytes were also cultured alone as an internal control. Cultured cells were exposed to anti-β_2GP1 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

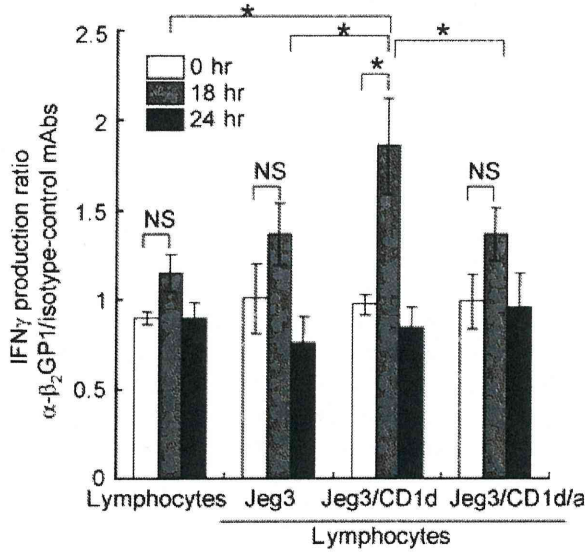

Fig. 8 Anti-β_2GP1 mAb enhances IFNγ secretion when added to co-cultures containing Jeg3/CD1d cells and decidual lymphocytes. IFNγ production in culture media collected for Fig. 7 was measured by ELISA and the production of IFNγ expressed as a ratio of anti-β_2GP1-exposed over control mAb-exposed specimens (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

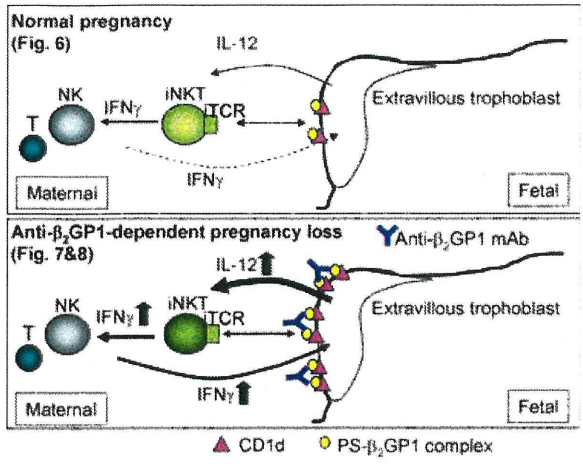

Fig. 9 Comparison of immune interactions at the human maternal–fetal interface during normal pregnancy with those proposed during anti-β_2GP1 mAb-related pregnancy loss. (Upper) In normal pregnancy, interaction of CD1d on the extravillous trophoblast cells (EVT) and maternal iNKT cells induces low level 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

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Productive lifecycle of human papillomaviruses that depends upon squamous epithelial differentiation

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Human papillomaviruses (HPVs) target the stratified epidermis, and can causes diseases ranging from benign condylomas to malignant tumors. Infections of HPVs in the genital tract are among the most common sexually transmitted diseases, and a major risk factor for cervical cancer. The virus targets epithelial cells in the basal layer of the epithelium, while progeny virions egress from terminally differentiated cells in the cornified layer, the surface layer of the epithelium. In infected basal cells, the virus maintains its genomic DNA at low-copy numbers, at which the viral productive lifecycle cannot proceed. Progression of the productive lifecycle requires differentiation of the host cell, indicating that there is tight crosstalk between viral replication and host differentiation programs. In this review, we discuss the regulation of the HPV lifecycle controlled by the differentiation program of the host cells.

Keywords: HPV, differentiation, epithelial cell, keratinocyte

INTRODUCTION

Human papillomavirus (HPV) infections of the anogenital organs are a very common "sexually transmitted disease (STD)." Although the incidence of cancer progression is low, a HPV infection is frequently detected in anogenital cancers. As for cervical cancer, HPV DNA is detected in more than 90% of cases. Approx. 5.5 million new cases of HPV infection are reported and there are c.a. 450,000 diagnoses of cervical cancer per year worldwide, leading to approximately 200,000 deaths each year, which ranks second among cancers in women (Parkin and Bray, 2006). HPV infections have also been associated with the head and neck squamous cell carcinomas (HNSCCs).

Human papillomavirus is categorized as a small virus containing DNA. More than 120 types of HPV have been identified and one-third of them target mucosal membranes, the remainder target the cutaneous membranes. Mucosa-tropic HPVs can be classified into two types based on their association with malignant carcinomas: a high-risk type (such as HPV type 16, 18, 31) and a low-risk type (such as HPV6 and 11; Howley, 1996). Prophylactic vaccines for HPV16 and18, Cervarix (GlaxoSmithKline), and for HPV6, 11, 16, and 18, Gardasil (Merck & Co.), have been developed recently and effectively prevent primary infections. They, however, cannot be used as therapeutic vaccines, indicating the importance of a Pap smear and the development of effective treatment strategies (Carter et al., 2011). In order to inhibit HPV-induced cancer, an understanding of the molecular basis of the infection and the characteristics of the infected lesions is important.

GENOME ORGANIZATION OF HPV AND FUNCTIONS OF VIRAL PROTEINS

Human papillomaviruses have a common gene organization (**Figure 1**): an early region encoding non-structural genes, the late region for structural genes, and a regulatory region (long control region: LCR).

The functions of each viral protein are summarized in **Table 1**. E1 and E2 are cooperatively involved in the initiation of viral DNA replication. E2 also functions as a transcriptional transactivator. E6 and E7 modulate the cell cycle control and contribute to viral genome maintenance (Frattini et al., 1996; Stubenrauch et al., 1998; Thomas et al., 1999). They also contribute to cancer development (Münger et al., 2004). Though E4 and E5 are speculated to modulate the productive phase of the HPV lifecycle, their biological roles remain unclear (Fehrmann et al., 2003; Genther et al., 2003; Nakahara et al., 2005; Wilson et al., 2005, 2007; Fang et al., 2006). Both L1 and L2 are capsid proteins.

HPV LIFECYCLE

The target of a HPV infection is the stratified epithelium. In the normal stratified epithelium, the cell attached to the basal membrane (basal cell) is the only cell that has the potential to proliferate. The basal cell divides into a new basal cell and a daughter cell that is detached from the basal membrane, and the daughter cell launches its differentiation process. The daughter cells exit from the cell cycle and change their gene expression pattern, proceeding to terminal differentiation, then peel off from the epithelium (Jones et al., 2007). The lifecycle of HPV is tightly regulated by the differentiation program of the host cells (**Figure 2**). In this section, the differentiation-dependent lifecycle of HPV is briefly summarized.

ENTRY OF HPV INTO THE BASAL CELLS OF STRATIFIED EPIDERMIS

Human papillomavirus virions invade through damaged areas of the epithelium and infect the basal cells. Although the receptor for the HPV infection has not been fully characterized, the following

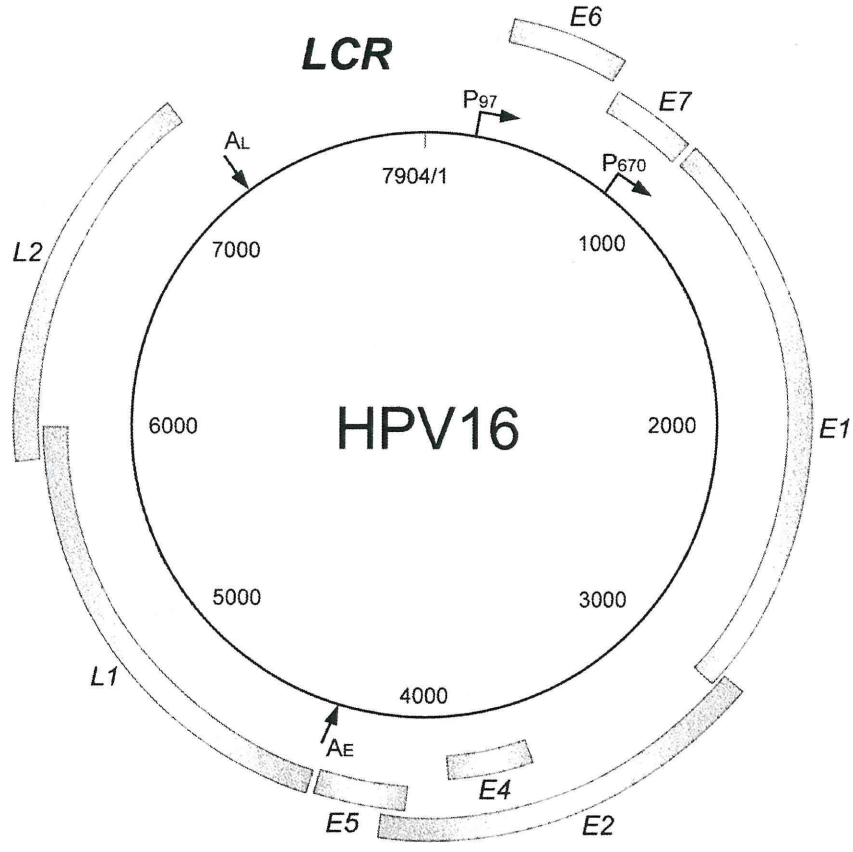

FIGURE 1 | The genome organization of HPV16. The HPV genome has a circular double-stranded DNA structure. The viral genes are transcribed in a single direction (clockwise). There are genes coding for non-structural proteins (E1, E2, E4, E5, E6, and E7) and structural proteins (L1, L2), and a transcriptional control region (long control region; LCR). LCR contains a DNA replication origin and functions as the regulator for the DNA replication. The major promoters and polyadenylation signals are indicated (P97, P670, AE, AL).

model has been postulated; virions initially attach to the heparan sulfate proteoglycan (HSPG) on the basal membrane, and transfer to the receptor expressed on the keratinocytes moving on the basal membrane in the wound-healing process, then enter the cells (Kines et al., 2009).

LOW-LEVEL EXPRESSION OF VIRAL GENES AND GENOME MAINTENANCE IN THE BASAL LAYER

Following viral entry and uncoating, HPV genomic DNA is transported into the nucleus and maintained at a low-copy number in the basal cells (50 ~ 100 copies per cell; in the basal layer, **Figure 2**; Moody and Laimins, 2010). Genome maintenance as episomal status is essential for the establishment of the early phase of the viral lifecycle (McBride et al., 2006).

PRODUCTIVE REPLICATION OF HPV IN THE DIFFERENTIATED CELLS

After leaving the basal membrane, the infected cells initiate the differentiation program. Because HPV does not encode DNA polymerase activity for viral genome replication, the host DNA replication machinery is required. However, the DNA replication activity is suppressed in the differentiated cells that exit from the cell division cycle. To ensure that the viral genome is replicated, HPV needs to reactivate cell division among the differentiation-initiated cells. E6 and E7 inactivate p53 and retinoblastoma protein (pRb), respectively, which enables the cells to maintain their DNA replication potential (Münger et al., 2004).

In the upper layers of the stratified epithelium (in the spinous layer, **Figure 2**), the expression of viral genes that are required for viral genome replication is markedly accelerated (Hummel et al., 1992; Ozbun and Meyers, 1997), inducing viral genome amplification to thousands of copies per cell (Bedell et al., 1991). Following the genome amplification, in the terminally differentiated cells, the synthesis of capsid proteins is triggered. The capsid proteins assemble into virions that encapsidate viral genomic DNA. The progenitor virions are released externally with peeled keratinocytes.

DIFFERENTIATION-DEPENDENT CONTROL OF HPV LIFECYCLE

The differentiation-dependent lifecycle of HPV is controlled of multiple levels, such as transcription, post-transcriptional processing, translation, and DNA replication. In the following sections, each regulatory mechanism is summarized.

Table 1 |

Function in viral lifecycle	Activities	Target factor
E1		
Replication of viral genome	DNA-binding activity, helicase activity, ATPase	RPA, topoisomerase, polymerase alpha-primase
E2		
Transcription of viral genes Replication of viral genome Maintenance of viral genome	Transactivation/transrepression, DNA-binding activity, DNA segregation in mitotic cell	Brd4, ChIR1
E6		
Reactivation of cellular replication mechanisms Proliferation, immortalization, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	**p53**, ADA3, p300/CBP, E6AP, SP1, c-Myc, NFX1-91, TERT, FAK, FADD, Caspase 8, BAX, BAK, IRF3, PDZ domain proteins
E7		
Reactivation of cellular replication mechanisms Proliferation, genomic instability, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	**RB**, p107, p130, HDAC, E2F6, p21, p27, CDK/cyclin, ATM, ATR, gamma-tubulin
E4		
?	Destruction of keratin network, induction of G_2M arrest of cell cycle	Cytokeratin 8/18
E5		
? Proliferation? Inhibition of apoptosis?	Affection of cellular signaling pathway	EGFR, PDGFR, V-ATPase, MHC1, TRAIL receptor, FAS receptor
L1		
Major capsid protein		
L2		
Minor capsid protein		

TRANSCRIPTIONAL REGULATION OF VIRAL GENES

Human papillomavirus has two major promoters, the early promoter and the late promoter. In HPV16, P97, and P670 have been identified as the early and late promoters, respectively (**Figure 1**). Transcriptional activity is mainly controlled by the LCR. A transcriptional enhancer is located within the LCR, with which various cellular transcription factors can associate (**Figure 3**).

The binding sites for the viral transcriptional regulator, E2, are found in HPV16 LCR. Viral gene expression is regulated by the occupancy status of the E2-binding sites (E2BSs; **Figure 3**), which is partly defined by the E2 expression level controlled by cellular differentiation status (Steger and Corbach, 1997; Hadaschik et al., 2003).

E2 functions in viral genome segregation by tethering the viral DNA to the mitotic chromatin, in which a cellular protein, bromodomain-containing protein 4 (Brd4), has been reported to be involved (McPhillips et al., 2006). Interaction between E2 and Brd4 is also required for the E2-mediated transcriptional activation and repression (McPhillips et al., 2006; Wu et al., 2012).

A ubiquitous transcription factor, Sp1 is a well-known regulator for HPV gene expression. The Sp1-binding site partially overlaps with one of the E2BSs (E2BS#2), and a TATA box element is located close to the promoter–proximal E2BS (E2BS#1; **Figure 3**). The binding of E2 to those E2BSs, therefore, interferes with the assembly of the transcriptional initiation complex, resulting in a suppression of E6/E7 expression that is governed by the early promoter activity (Tan et al., 1992). It was also reported that Sp1 altered the chromatin structure of HPV16 LCR, offering the accessibility of transcription factors (Stünkel and Bernard, 1999).

TRANSCRIPTIONAL CONTROL IN THE UNDIFFERENTIATED CELLS

Transcripts of viral early genes are expressed in the infected basal cells, which is essential for the viral DNA replication (Dürst et al., 1992). It was reported that a unique promoter, P14, was utilized for E1 expression and the E2BSs were considered as necessary for the P14 activity (Lace et al., 2008). The transcript initiated from P14 is a poly-cistronic mRNA containing E6, E7, and E1, in which the shunting in ribosomal scanning process enables the translation of E1 (Remm et al., 1999). The regulatory mechanism for E2 expression has not been clarified. The early promoter is used for E6 and E7 expression, in which several transcription factors, including AP-1, glucocorticoid receptor, NF1, Oct-1, Sp1, YY-1, and CDP, are involved (**Figure 3**; Desaintes and Demeret, 1996).

TRANSCRIPTION IN THE DIFFERENTIATED CELLS

The early promoter is activated in association with the differentiation process, increasing the E1/E2 expression (Hummel et al., 1992; Ozbun and Meyers, 1997). Although levels of E6 and E7 also

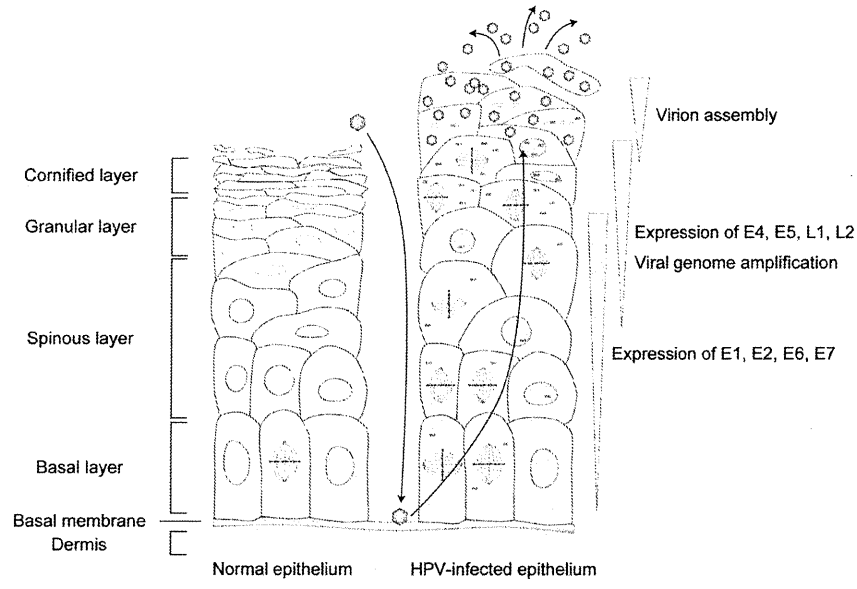

FIGURE 2 | The lifecycle of human papillomaviruses (HPVs). HPVs infect specifically the cells in the basal layer of the stratified epithelium through lesions. Viral genomes are maintained as episomal DNA in the nuclei of infected cells. The viral lifecycle is strictly controlled by host cell differentiation, and the late lifecycle (productive lifecycle) occurs in upper layers of the epithelia that are terminally differentiated, and the progenitor virions are released from the cornified keratinocytes.

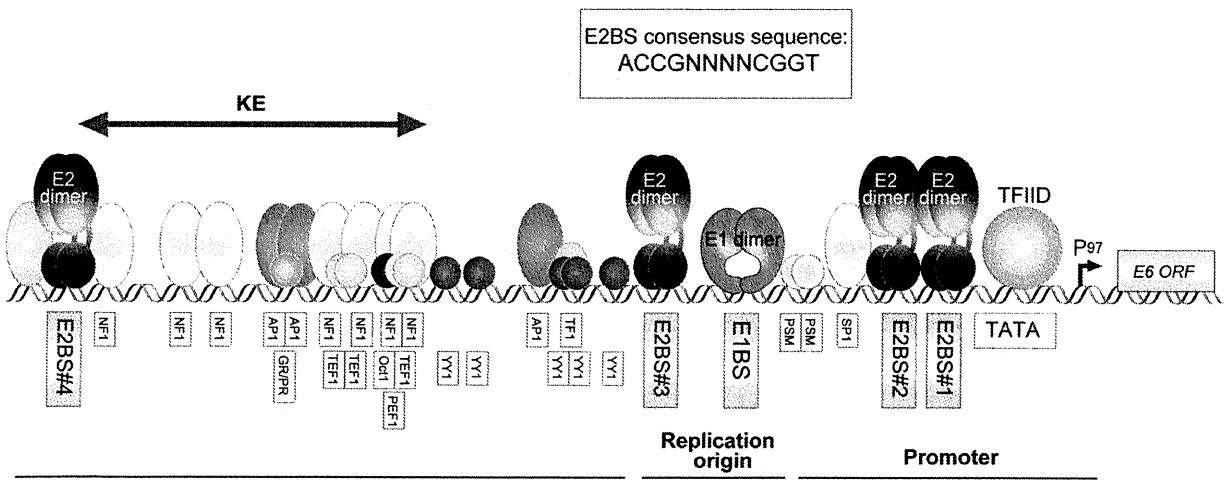

FIGURE 3 | The structure of HPV16 LCR (region of the control of early promoter P97). The early promoter P97 and replication origin are located in LCR, which are regulated by various cellular factors. Activity of P97 is regulated by AP-1, NF1, SP1, TFIID, TF1, Oct-1, PSM, and the viral transcription factor E2. Four E2-binding sites (E2BS) have been identified in HPV16 LCR and the consensus sequence for E2BS is shown in an inset. A glucocorticoid receptor and progesterone receptor (GR/PR) recognition element was also identified in the LCR. The existence of a keratinocyte-specific enhancer (KE) has been proposed (Desaintes and Demeret, 1996).

increase with the early promoter's activation, the E2 overexpressed in the upper layer is thought to suppress their transcription via the mechanism mentioned above. E6 and E7 are important in maintaining infected cells in an undifferentiated state, but terminal differentiation is required for the productive replication of HPV. The inhibition of E6/E7 expression by E2 might promote cellular differentiation, and the cells undergo terminal differentiation, which is suitable for the viral productive lifecycle.

AP-1, a heterodimer composed of Fos and Jun, is considered to be involved in the differentiation-dependent transcriptional

control in keratinocytes; there are reports that the expression profiles of Fos and Jun family members were modified, and that the interaction between AP-1 and KRF-1, a keratinocyte-specific transcription factor (Mack and Laimins, 1991), was strengthened in the differentiation process (Desaintes and Demeret, 1996; Thierry, 2009). Several transcriptional factors were reported to be involved in the differentiation-dependent control of LCR function; EPOC-1/Skn-1a, C/EBP-α, -β, c-Myb, NF1, NFATx, Pax5, and WT1 (Desaintes and Demeret, 1996; Thierry, 2009).

The late promoter is specifically activated in the differentiated layers of epithelium. The late promoter activity is suppressed by CDP (CCAAT displacement protein) and YY-1, whose binding potential was reported to be decreased in differentiated keratinocytes (Ai et al., 1999, 2000). There was also a report that the expression ratio of a transcription factor, Sp1 and its antagonist, Sp3, was altered through the differentiation, which activated the late promoter activity (Apt et al., 1996). The binding of hSkn-1a and C/EBPα to the proximal region of the late promoter contributes to the control of the late promoter activity (Kukimoto and Kanda, 2001; Wooldridge and Laimins, 2008). The involvement of E7 in the regulation of the late promoter activity was also described (Bodily and Laimins, 2011; Bodily et al., 2011). It still remains necessary to clarify the regulatory mechanism for the late promoter in the differentiation of epithelial cells.

METHYLATION OF THE HPV GENOME DURING THE CELL DIFFERENTIATION PROCESS

HPV gene expression is controlled by the methylation of HPV genomic DNA. As E2BSs contain CpG dinucleotides (see inset in **Figure 3**), they can be modified by DNA methylation in the host cell. E2BSs are reported to be highly methylated in undifferentiated cells, inhibiting E2-binding, and demethylation at the E2BSs occurs in association with the cell differentiation (Kim et al., 2003; Vinokurova and von Knebel Doeberitz, 2011).

RNA PROCESSING

For conversion of the gene expression profile from the early to late phase of viral replication, RNA processing is considered critical. The primary transcript of HPV encodes multiple viral genes, and precise RNA processing is essential to produce the mRNA for each viral gene at an appropriate stage of cell differentiation (Schwartz, 2008).

In the early phase of the viral lifecycle, the primary transcription initiated by the early promoter is terminated at the early poly(A) signal, AE (**Figure 1**), and the transcript is processed by using the early splicing signals, which produces the mRNAs encoding the viral early genes. In the differentiated cells, the transcripts for the late genes are expressed from the late promoter and utilize a late poly(A) signal, AL (**Figure 1**), and late splicing signals. The early and late splicing signals compete for the splicing factors, so their usages are generally exclusive.

Multiple splicing signals are found in the HPV genome, which are utilized for the expressions of various viral genes (**Figure 4**). These splicing signals can be categorized into three groups; early phase-specific signals (DS226, SA409, SA526, SA742 in HPV16), late phase-specific signals (SD 3632 and SA5639), and non-specific signals (SD880, SA2709, SA3358; Schwartz, 2008).

Early splicing events have three major roles; regulation of the expression ratio of early genes, production of splicing variants of viral genes, and suppression of late gene expressions. The early

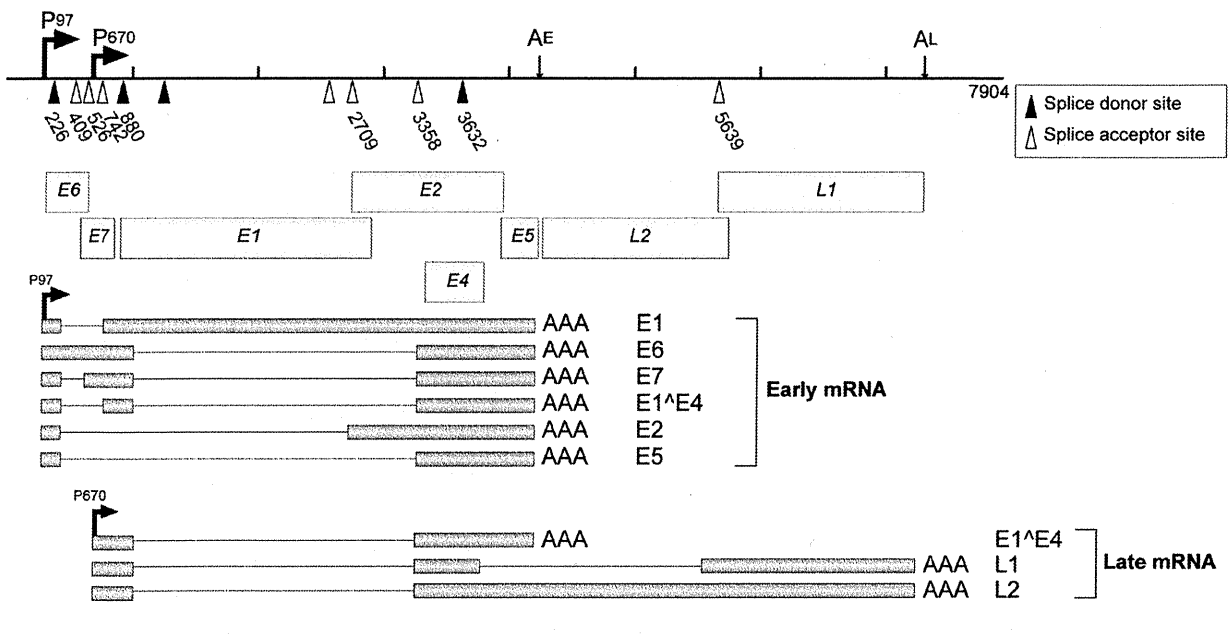

FIGURE 4 | The splicing signals and the transcripts of HPV16. P97 and P670 are the early and late promoters, respectively, for HPV16. AE and AL indicate the early and late polyadenylation signals, respectively. Open triangles indicate splicing acceptors and filled triangles indicate splicing donors. Major mRNAs and their products are indicated. This figure is cited from a review (Schwartz, 2008).