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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

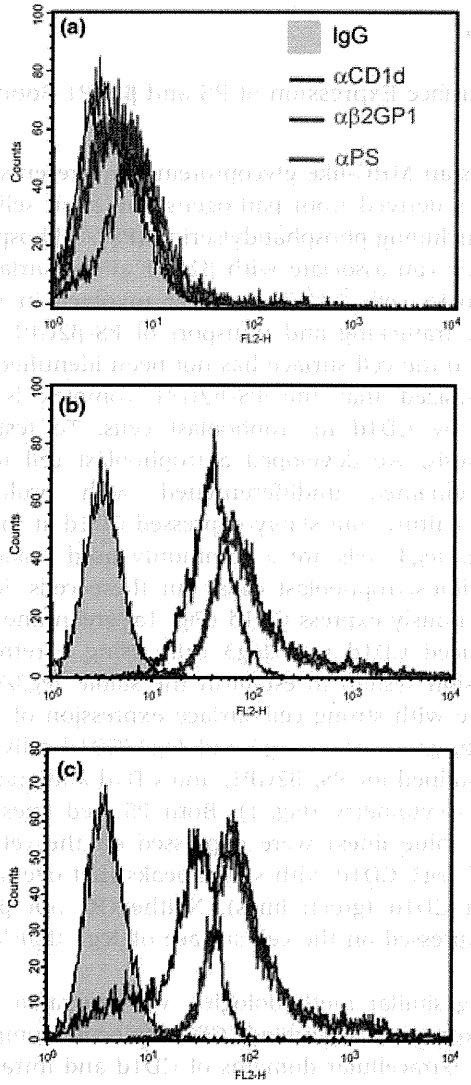

Fig. 1 Cell-surface expression of PS and β_2GP1 is CD1d-dependent. Jeg3 (a), Jeg3/CD1d (b) and Jeg3/CD1d/a (c) cells were triple stained with the anti-CD1d mAb (green), the anti-PS Ab (red) and anti-β2GP1 mAb (blue) and analyzed using flow cytometry. Background staining with isotype-matched control antibody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

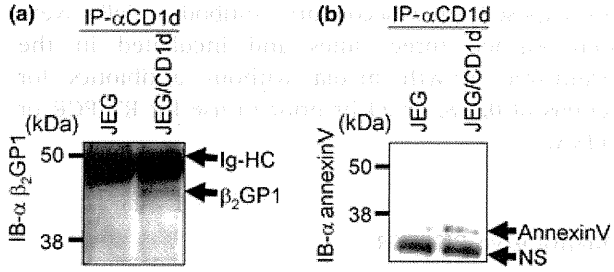

Fig. 2 The PS-β_2GP1 complex binds to CD1d. An anti-CD1d mAb was used to immunoprecipitate CD1d from Jeg3 or Jeg3/CD1d total cell lysates. (a) Immunoprecipitants were immunoblotted with an HRP-labeled anti-β_2GP1 mAb to detect β2GP1. An extra-band at 42 KDa, representing β_2GP1, was observed only in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

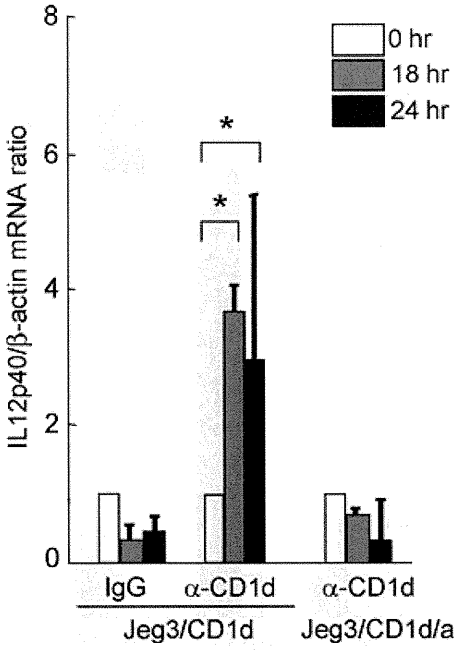

Fig. 3 CD1d cross-linking using anti-CD1d primary and anti-Ig secondary antibodies induces IL12 transcription from Jeg3/CD1d cells but not Jeg3/CD1d/a cells. Jeg3/CD1d cells or Jeg3/CD1d/a were exposed in culture to 10 μg/mL of the anti-CD1d mAb (51.1) or an isotype-control mAb for 1 hr. After washing,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

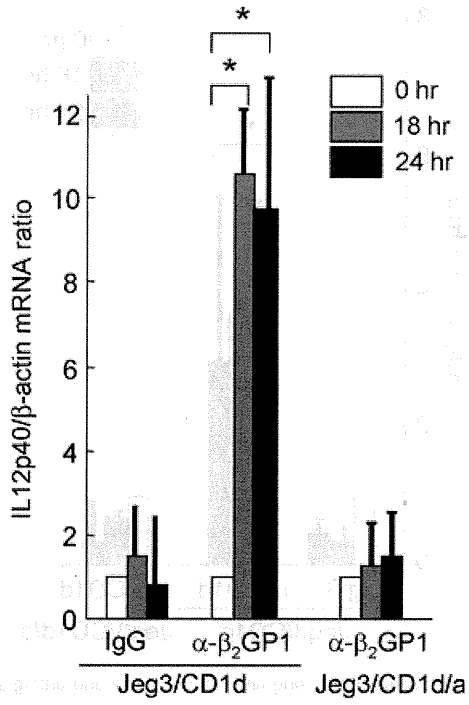

Fig. 4 Ligation of CD1d/PS/β_2GP1 complexes on Jeg3/CD1d cells using only anti-β_2GP1 mAbs increases IL12 transcription. Anti-β2GP1 and isotype-matched control mAbs were used as cross-linking antibodies in the absence of a secondary cross-linking reagent. Cells were harvested at 0, 18, and 24 hrs after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

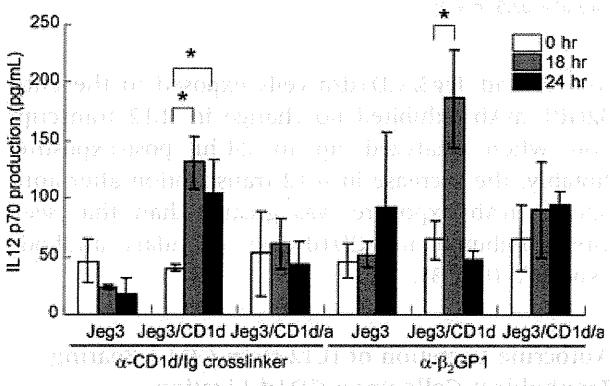

Fig. 5 Ligation of CD1d on Jeg3/CD1d cells using either anti-CD1d or anti-β_2GP1 mAbs increases IL12 secretion from Jeg3/CD1d cells. After cross-linking by anti-CD1d or anti-β_2GP1 mAb as shown in Fig. 4, cells were incubated in serum-free growth media without antibiotics for periods of 0–24 hrs. Autocrine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

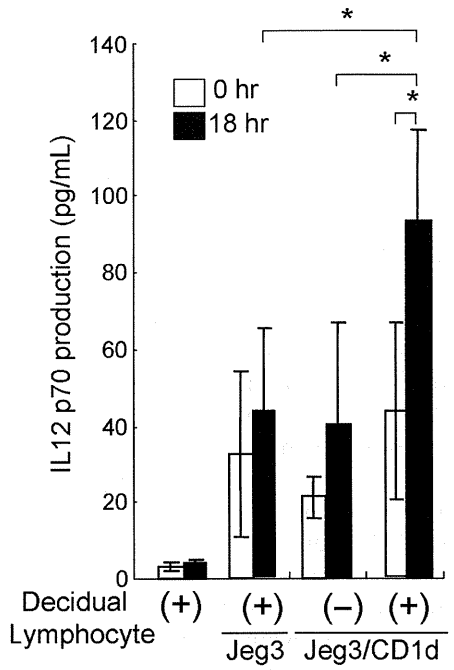

Fig. 6 IL12 secretion from Jeg3/CD1d cells is induced by the presence of decidual lymphocytes that include CD1d-restricted iNKT cells. Decidual lymphocytes were isolated from normal human decidual tissues collected at 7–9 gestational weeks. 10^5 decidual lymphocytes were cultured with Jeg3 or Jeg3/CD1d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

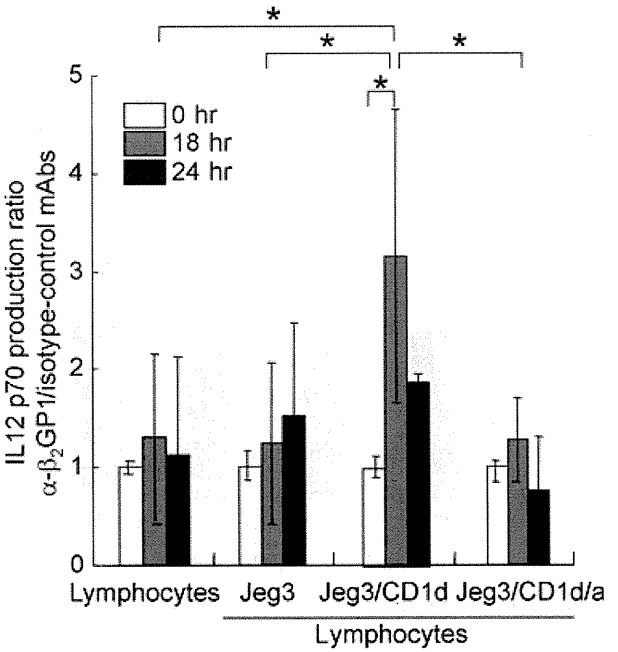

Fig. 7 Anti-β_2GP1 mAb enhances IL12 secretion during co-culture of Jeg3/CD1d cells and decidual lymphocytes. Decidual lymphocytes were co-cultured with Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells. Decidual lymphocytes were also cultured alone as an internal control. Cultured cells were exposed to anti-β_2GP1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

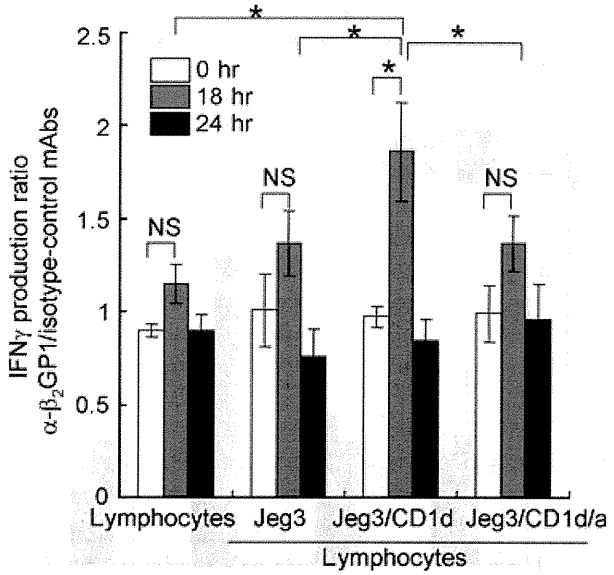

Fig. 8 Anti-β_2GP1 mAb enhances IFNγ secretion when added to co-cultures containing Jeg3/CD1d cells and decidual lymphocytes. IFNγ production in culture media collected for Fig. 7 was measured by ELISA and the production of IFNγ expressed as a ratio of anti-β_2GP1-exposed over control mAb-exposed specimens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

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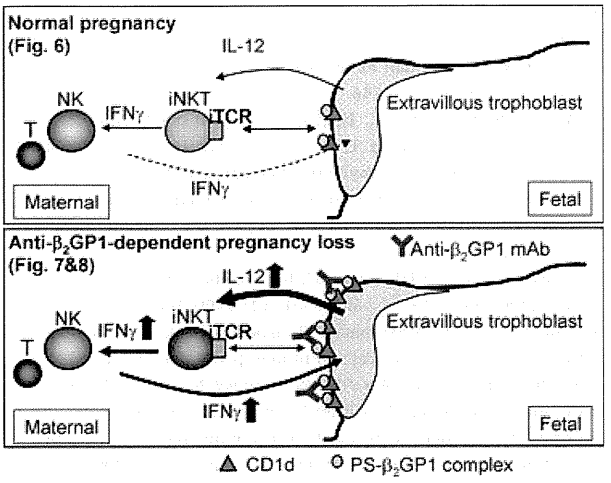

Fig. 9 Comparison of immune interactions at the human maternal–fetal interface during normal pregnancy with those proposed during anti-β_2GP1 mAb-related pregnancy loss. (Upper) In normal pregnancy, interaction of CD1d on the extravillous trophoblast cells (EVT) and maternal iNKT cells induces low level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

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

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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.

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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

issue. The bivalent vaccine was officially approved for use in Japan by the end of 2009, but there has been an ongoing debate on whether the use of HPV vaccine should be underpinned by tax-payers' money and if so, how much the government should spend.[9]

Despite its proven cost-effectiveness in other settings[10–12] a simple extrapolation of the costs and effectiveness of HPV vaccine in countries other than the Japanese setting is not appropriate because of the differences in cervical cancer epidemiology and health systems. The prevalence of HPV types differs between geographic regions. In the case of squamous cell carcinoma, HPV16 was the predominant type (46–63%) followed by HPV18 (10–14%), 45 (2–8%), 31 (2–7%) and 33 (3–5%) in all regions except Asia, where HPV types 58 (6%) and 52 (4%) were more frequently identified.[13] In Japan, HPV52 and HPV58 are most frequently found in squamous intraepithelial lesion following HPV16.[14] A relatively lower prevalence of HPV16 and HPV18 in Japan has cast doubt on the effectiveness of the current HPV vaccine when compared with other countries.[15]

Only one study has evaluated the cost-effectiveness of HPV vaccination in the Japanese setting.[16] However, the study did not compare strategies with a variable screening rate. Nor did it consider the effect of HPV type prevalence by age in Japan. Therefore, a cost-effectiveness analysis of screening coverage and vaccination, taking into account the age-specific prevalence by HPV type in the Japanese setting is urgently needed to inform and support policy decisions. Healthcare resources are limited; resources dedicated to screening and vaccination are no longer available for alternative healthcare uses and therefore the chosen strategy should represent a cost-effective use of scarce resources. The major objective of the present study is to assess the cost-effectiveness of universal vaccination against HPV in Japan from a societal perspective where the coverage of Pap smears is low and HPV oncogenic types are different from in other settings.

Methods

Natural history model of HPV infection

We developed a state-transition Markov model that simulates the natural history of HPV infection and carcinogenesis, in which transitions take place from one state to another at 1-month intervals (Figure 1). The model has 25 Markov states. The entry point into the model is girls aged 11 years with no previous exposure to HPV. We assumed that when girls/women enter the model, they start sexual activities, so acquiring a risk for HPV with the currently observed probabilities. In each cycle, they proceed to one of the four states: HPV16 and 18 DNA-positive group (HPV16 and 18), the other high-risk HPV DNA-positive group (other HR), the low-risk HPV DNA-positive group (LR), and the non-infected group (Normal) using monthly transition probabilities based on the systematic review of published literature.[17]

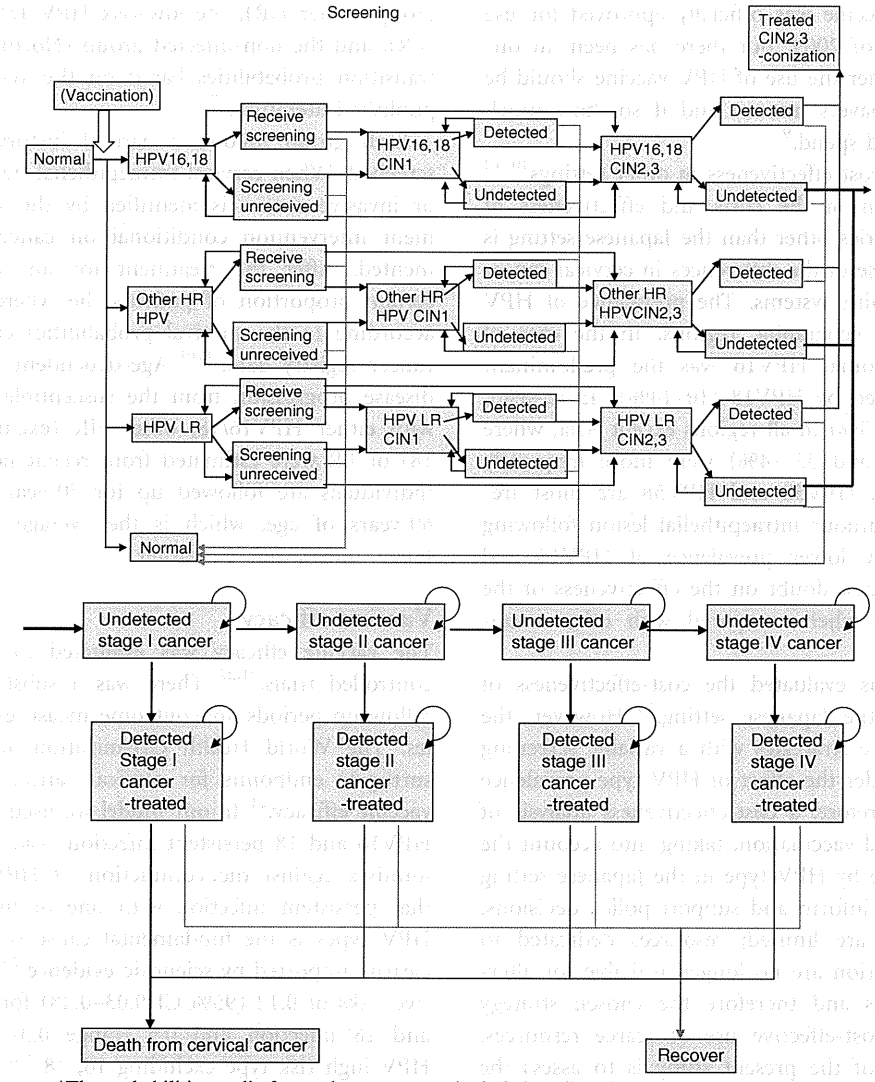
Each group follows a natural history unless they are screened. When cervical intraepithelial neoplasia (CIN) 2, 3 or invasive cancer is identified by the screening, a treatment intervention conditional on cancer stage is implemented. After the treatment for an invasive cancer, a certain proportion of patients die whereas others survive according to the survival probabilities compiled from the cancer registry data.[18,19] Age-dependent transition rates of disease progression from the susceptible to those infected with either HPV16/18, other HR (excluding HPV16 and 18) or LR were estimated from recent Japanese data.[20] All individuals are followed up for 50 years until they reach 60 years of age, which is the average retirement age in Japan, unless they die earlier.

Vaccine efficacy

The vaccine efficacy was evaluated in eight randomised controlled trials.[21,22] There was a substantial variation in follow-up periods and outcome measures among the studies. The World Health Organization adopted CIN2/3 as surrogate endpoints for cervical cancer in trials assessing vaccine efficacy.[21] In our model we used the relative risk of HPV16 and 18 persistent infection risk, as the vaccine immunises against the contraction of HPV. The hypothesis that persistent infection with one of the 15 carcinogenic HPV types is the fundamental cause of cervical cancer is clearly supported by scientific evidence.[23] We assumed relative risks of 0.12 (95% CI 0.03–0.48) for persistent HPV16 and 18 infection and 0.5 (range 0.3–0.7) for persistent HPV high-risk type excluding 16, 18.[21,22,24,25] Additionally, we assumed 100% lifetime protection against HPV16 and 18 once fully vaccinated.

Intervention strategies

The bivalent vaccine was approved for use in women and girls who are over 10 years old. The Japan Society of Obstetrics and Gynaecology recommended administering HPV vaccine among girls from 11 to 14 years of age as a priority, partly because they are old enough to understand the meaning of the vaccination and partly because the vaccination in this age group is efficient and ensures early protection against HPV with high immunogenicity. Therefore, for strategies which include vaccination, all 11-year-old girls are vaccinated at the entry point into the model. We assumed that there is no exposure to any HPV types before the entry to the model. At 20 years of age, they start receiving screening every 2 years according to the current Japanese recommendations.[5] Our reference strategy is the screening programme only with the current level of



*The probabilities to die from other causes are included at all each states.

Figure 1. HPV natural history model.

Table 1. Strategies

1. 20% coverage rate of screening with no vaccination
2. 50% coverage rate of screening with no vaccination
3. 80% coverage rate of screening with no vaccination
4. 20% coverage rate of screening with vaccination for all 11-year-old girls
5. 50% coverage rate of screening with vaccination for all 11-year-old girls
6. 80% coverage rate of screening with vaccination for all 11-year-old girls

coverage (i.e. 20%).[6,7] Table 1 summarises six strategies that were analysed in the present study. The sensitivity of the Pap smear was assumed to be 94.7% as previously reported.[26] The specificity (reported to be 98.9%) is not included in the model because screening will be repeated in false positives as determined by the cytology results.

Survival rates of women with cervical cancer

We used the data from life tables of Japanese vital registration to estimate the population-based mortality rates by age from cervical cancer and other competing risks.[27] Cumulative nationwide survival rates by cancer stages of FIGO classification were not available in Japan. We adopted the data from the US SEER programme (Surveillance Epidemiology and End Results),[18,19] which were calibrated using data from an existing Japanese regional cancer registry.[28]

Transition probabilities

Several natural history models of HPV have been developed and used in policy evaluations.[29,30] Whereas a particular

parameter has been common to several natural history models, there is a huge variation in the structure and parameters used in the previous models.[29] We used age-dependent type-specific HPV prevalence data from Japanese women[20] to derive transition probabilities from the susceptible to those infected with HPV16/18, other HR types and LR types.

Other model parameters were estimated from systematic literature reviews and then calibrated to the Japanese setting (Table 2).[17] We simulated the model by using the transition rates of CIN2, 3 to the undetected stage I cancer of HPV16/18 and other HR depending on their age groups in Japan. Then we adjusted them by using the data of age-dependent incident rates of cervical cancer. We validated the model by goodness-of-fit statistics using age-dependent mortality rates of cervical cancer.

Table 2. Transition rates

Variable	Baseline values	Range
Progression		
HPV DNA to CIN1		
Low-risk HPV	0.0264	0.0245–0.0284
High-risk 16, 18 HPV	0.0150	0.0026–0.0274
High-risk other HPV	0.0376	0.0271–0.0480
HPV DNA to CIN2, 3		
Low-risk HPV	0.00003	0.000003–0.00006
High-risk 16, 18 HPV	0.0012	0.000014–0.0024
High-risk other HPV	0.000025	0.000002–0.00005
CIN1 to CIN2, 3		
Low risk HPV	0.0003	0.00002–0.0005
High-risk 16, 18 HPV	0.0042	0.0001–0.0082
High-risk other HPV	0.0015	0.0001–0.0028
CIN2, 3 to undetected stage 1 cancer		
High-risk 16, 18 HPV	0.0049*	0.00001–0.0098
High-risk other HPV	0.0088*	0.00004–0.0176
Progression rates in unscreened women with cancer		
Stage I to stage II	0.0188	
Stage II to stage III	0.0250	
Stage III to stage IV	0.0375	
Regression		
HPV DNA to Normal		
Low-risk HPV	0.1951	
High-risk 16, 18 HPV	0.1951	
High-risk other HPV	0.1951	
CIN1 to Normal		
Low-risk HPV	0.0854	
High-risk 16, 18 HPV	0.1406	0.1316–0.1497
High-risk other HPV	0.0430	
CIN2, 3 to Normal (70% of women)		
Low-risk HPV	0.0145	0.0052–0.0238
High-risk 16, 18 HPV	0.0045	0.0010–0.0080
High-risk other HPV	0.0082	0.0029–0.0134
CIN2, 3 to HPV DNA or to CIN1 (15% of women each)		
Low-risk HPV	0.0031	0.0011–0.0051
High-risk 16, 18 HPV	0.0010	0.0002–0.0017
High-risk other HPV	0.0018	0.0006–0.0029

*Multiplied by age-dependent rate derived from calibration.

Cost estimation

A societal perspective was adopted for this cost analysis. Cost estimates are presented in Table 3 that include programme costs and time costs. We approximated the programme costs by using the current national tariff used by the national health insurance scheme.[31] These data were cross-validated by the cost of treatments and care for gynaecological patients at the University of Tokyo Hospital between August 2007 and November 2009. Both variable costs and doctor's fees are included in the programme costs according to the fee schedule set by the national tariff.[31] We estimated patients' time cost by using the national average hourly wage of part-time workers from a national survey.[32]

Cost-effectiveness analysis

We calculated quality-adjusted life-years (QALYs) from the model outputs on incidence, duration and mortality. The quality-of-life weights for different health states were based on those used in previous studies (Table 4).[33–35] All costs and benefits were discounted at 3%, a frequently used rate for cost-effective analysis done in Japanese settings.[36]

In line with a standard health economic evaluation, strategies are ranked in order of effectiveness after excluding dominated strategies.[37] Incremental cost-effectiveness ratios (ICERs) are then calculated for each strategy relative to the next best alternative. The preferred strategy is the most effective strategy with an ICER within the willingness to pay threshold of 4.5 million yen. A commonly applied threshold for acceptable cost-effectiveness in the USA is $50,000;[38] it is often used as a basis of cost-effective analysis in a Japanese setting.

Table 3. Cost data

Costs involving patient's time costs	Yen
Screening visit (Pap-test) per event	7460
CIN1 detected patient per month	4228
CIN2, 3 detection per event	28,360
Conisation cost per case	310,900
Treatment cost for stage I cancer case	664,300
Treatment cost for stage II cancer per case	2,869,900
Treatment cost for stage III cancer per case	3,066,500
Treatment cost for stage IV cancer per case	2,940,200
Average monthly wage for a Japanese case	226,100
Vaccination cost (for three doses/visits)	58,000

Table 4. Quality of life weights

Variable	Baseline values	Range
Quality of life weights for CIN		
CIN1	0.97	0.97–1.00
CIN2, 3	0.93	0.93–1.00
Quality of life weights for invasive cancer		
Stage I	0.65	0.49–0.81
Stage II	0.56	0.42–0.70
Stage III	0.56	0.42–0.70
Stage IV	0.48	0.36–0.60
Quality of life weights after treatment for invasive cancer		
Stage I	0.97	0.73–0.99
Stage II	0.9	0.68–0.98
Stage III	0.9	0.68–0.98
Stage IV	0.62	0.47–0.78

Results

Reduction in lifetime risk of cancer

Figure 2 shows the lifetime risk of cervical cancer by strategy estimated from a two-dimensional probabilistic sensitivity analysis. The range represents the minimum and maximum numbers of cervical cancer incidence per 100 000 population and its interquartile range (IQR). The bars represent the median value. Increasing the coverage of screening from the current level of 20–50 and 80% will substantially reduce the number of incident cervical cancer cases by 45.5% (IQR 42.0–48.7) and 63.1% (IQR 60.5–65.7), respectively. Combined strategies of 20, 50 and 80% screening coverage rate yields, respectively, a 66.1% (IQR 68.3–64.2), 80.9% (IQR 78.6–83.3) and 86.8% (IQR 85.4–87.9) reduction in cervical cancer incidence.

Total costs and QALYs of vaccination and screening programmes

Total QALYs gained per 100 000 population for each strategy showed slight increase as the screening coverage increases and the universal vaccination is added (Figure 3). Figure 4 shows cost per person for each strategy. The squares represent average values and the range represents average value ± 2 SD. Costs of strategies including vaccination are approximately four times higher than that of strategies without vaccination. Increasing the screening coverage rate was cheaper than introducing vaccination for all 11-year-old girls.

Incremental cost-effectiveness ratio

Table 5 shows the ICER of each strategy compared with its next best alternative strategy. Using the default model values, 50% screening coverage with a vaccination strategy was the most cost-effective when using a willingness to pay for a QALY threshold of 4,500,000 yen (≅ US$500,000) (Figure 5).

Sensitivity analysis on vaccine efficacy

We performed a sensitivity analysis on vaccine efficacy. The vaccine efficacy is determined by the combination of risk ratios to acquire HPV16/18 and other HR in our model. Table 6 shows cost and QALYs derived from the reference vaccine efficacy, minimum and maximum vaccine efficacy per 1000 people. Differences in vaccine efficacy would result in the differences in programme costs ranging from approximately 4,000,000–8,000,000 yen (≅US$480,000–960,000).

Table 7 shows the ICERs derived from the sensitivity analysis. The current strategy is dominated by strategies with a higher screening rate. A screening rate of 20% with a vaccination strategy is always ruled out because of extended dominance. The ICER for a screening rate of 50 and 80% with vaccination strategies was sensitive to the

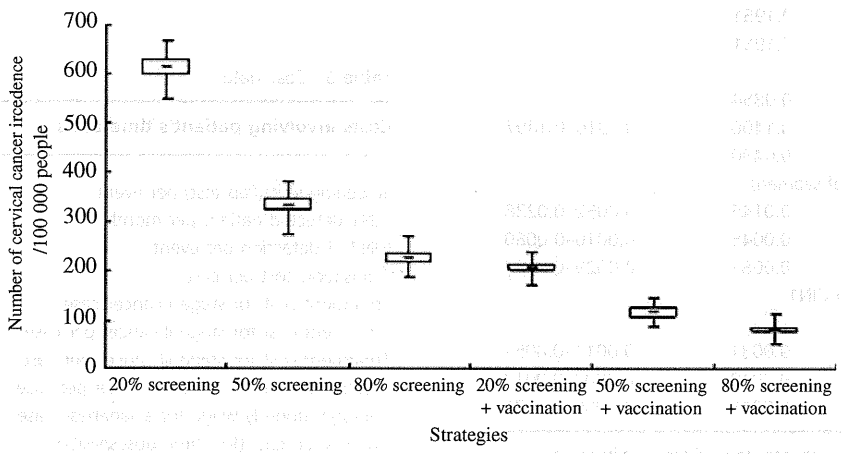


Figure 2. Lifetime risk of cancer for each strategy.

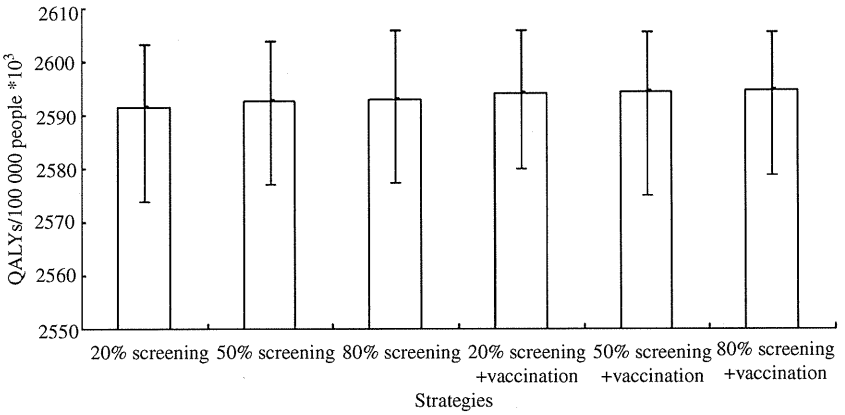


Figure 3. Total QALYs per 100,000 people for each strategy.

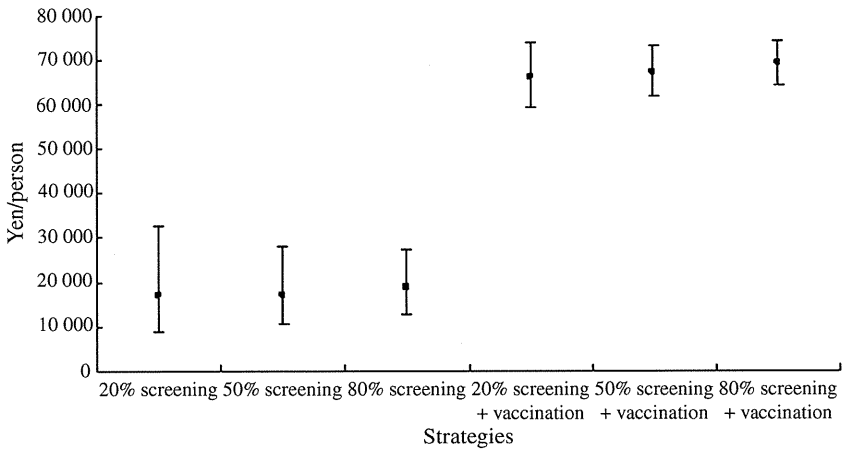


Figure 4. Cost per person for each strategy.

Table 5. Cost effectiveness of alternative screening and vaccination strategies

Strategy	Incremental cost effectiveness Ratio* (Yen/QALY)	
20% Screening	–	Dominated
50% Screening	658	
80% Screening	571 015	
20% Screening + vaccination	–	Extended Dominance
50% Screening + vaccination	2 920 636	
80% Screening + vaccination	8 568 182	not cost effective

*Ratio of additional costs and benefits of a particular strategy compared with the previous strategy.

differences in incremental costs and effectiveness given by the result of a two-dimensional probabilistic sensitivity analysis of the model with each vaccine efficacy. With the

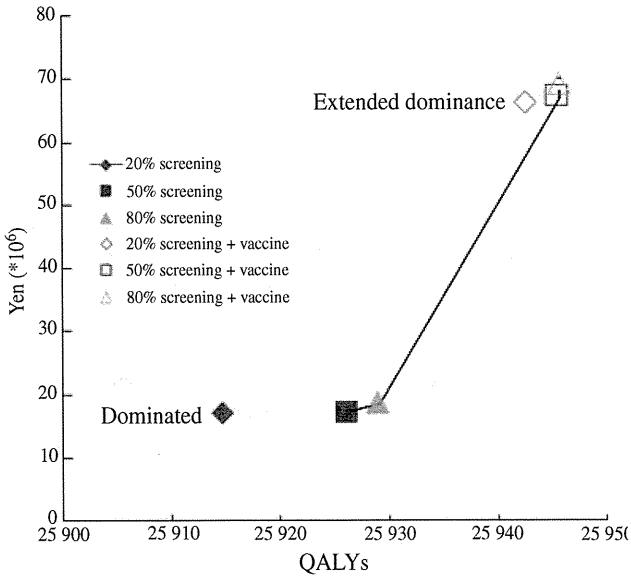


Figure 5. Cost and QALYs per 1000 people.

Table 6. Costs and QALYs per 1000 people of varied vaccine effect

Strategy	Minimum vaccine effect*		Baseline vaccine effect*		Maximum vaccine effect*	
	Cost (¥)	QALYs	Cost (¥)	QALYs	Cost (¥)	QALYs
Screening 20% + vaccination	69,561,000	25 933.88	66,114,000	25 942.64	62,628,000	25 950.77
Screening 50% + vaccination	70,300,000	25 937.33	67,334,000	25 945.54	64,300,000	25 953.22
Screening 80% + vaccination	72,129,000	25 940.81	69,219,000	25 945.76	66,277,000	25 953.07

*Minimum vaccine effect means relative risks of 0.48 for persistent HPV16 and 18 infection and 0.7 for persistent HPV high-risk type excluding 16, 18 infection. Baseline vaccine effect means relative risks of 0.12 for persistent HPV16 and 18 infection and 0.5 for persistent HPV high-risk type excluding 16, 18 infection. Maximum vaccine effect means relative risks of 0.03 for persistent HPV16 and 18 infection and 0.3 for persistent HPV high-risk type excluding 16, 18 infection.

Table 7. Sensitivity analysis on vaccine effect (ICER)*

Strategy	Minimum vaccine effect**	Baseline vaccine effect**	Maximum vaccine effect**
Screening 20%	Dominated	Dominated	Dominated
Screening 50%	658	658	658
Screening 80%	Extended dominance	571 015	571 015
Screening 20% + vaccination	Extended dominance	Extended dominance	Extended dominance
Screening 50% + vaccination	Extended dominance	2 920 636	1 874 867
Screening 80% + vaccination	3 745 442	8 568 182	Dominated

*Incremental cost effectiveness ratio (Yen/QALY).
**Minimum vaccine effect means relative risks of 0.48 for persistent HPV16 and 18 infection and 0.7 for persistent HPV high-risk type excluding 16, 18 infection. Baseline vaccine effect means relative risks of 0.12 for persistent HPV16 and 18 infection and 0.5 for persistent HPV high-risk type excluding HPV16, 18 infection. Maximum vaccine effect means relative risks of 0.03 for persistent HPV16 and 18 infection and 0.3 for persistent HPV high-risk type excluding 16, 18 infection.

minimum efficacy, a combined strategy of 80% screening and universal vaccination is most cost-effective. On the other hand, with the maximum and baseline vaccine efficacy, a combined strategy of 50% screening and universal vaccination remains most cost-effective.

Discussion

The introduction of HPV vaccine to the Japanese population has been controversial because the coverage of Pap smear screening is low and the prevalence of HPV types is different from that observed in Western countries.

To date there has been only one study that has assessed the impact of introducing HPV vaccine in Japan.[16] However, this study suffered from several major limitations. It did not distinguish health status related to HPV type 16 and 18 from other high-risk types. We modelled the natural history of each HPV type status; HPV16/18, other HR, and LR. We used different vaccine efficacies depending on the HPV types with a range that was derived from a meta-analysis of the available evidence. The previous study also did not include strategies of varied screening rates without vaccination. The authors analysed the effect of screening atthe currently observed levels ranging from 13.6 to 24.7%, and so the impact of increasing Pap smear coverage was not considered. Instead, the present study compared the strategies of varied screening rates ranging from 20 to 80%.

Our analysis suggests that increasing cervical cancer screening coverage to 50% would halve the incidence of cervical cancer and save programme costs and that the introduction of HPV vaccination would reduce the incidence by two-thirds but result in a four-fold increase in programme costs. Using the model's default values, a combined strategy to expand the coverage of cancer screening up to 50% and the introduction of universal vaccination would be most cost-effective. The results are robust with sensitivity analysis in which the optimum coverage level most likely lies somewhere between 50 and 80%. Our result confirms the need for expanding coverage for Pap smears in Japan as previously suggested,[39] to maximise the impact of the cervical cancer strategy regardless of whether a national vaccine programme is also implemented.