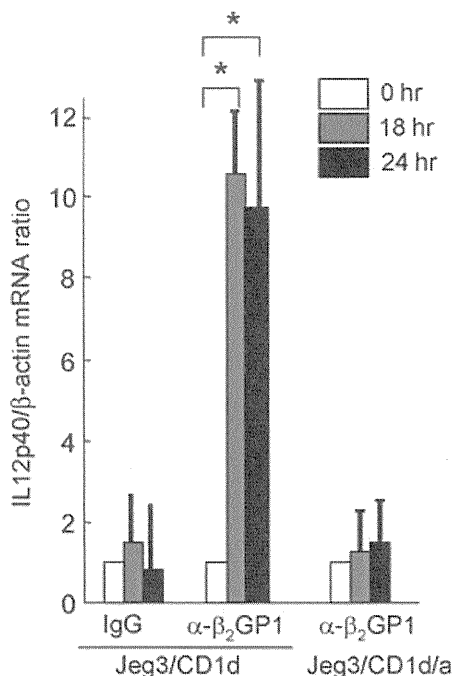

Fig. 4 Ligation of CD1d/PS/β_2GP1 complexes on Jeg3/CD1d cells using only anti-β_2GP1 mAbs increases IL12 transcription. Anti-β2GP1 and isotype-matched control mAbs were used as cross-linking antibodies in the absence of a secondary cross-linking reagent. Cells were harvested at 0, 18, and 24 hrs after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

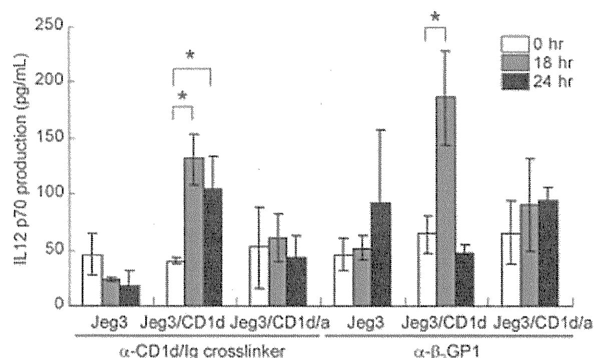

Fig. 5 Ligation of CD1d on Jeg3/CD1d cells using either anti-CD1d or anti-β_2GP1 mAbs increases IL12 secretion from Jeg3/CD1d cells. After cross-linking by anti-CD1d or anti-β_2GP1 mAb as shown in Fig. 4, cells were incubated in serum-free growth media without antibiotics for periods of 0–24 hrs. Autocrine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

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

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

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

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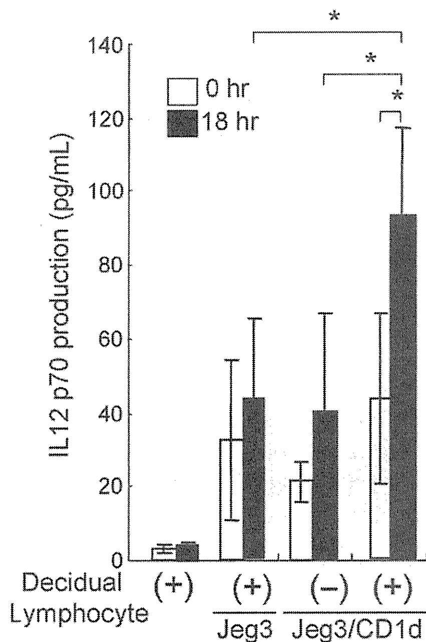

Fig. 6 IL12 secretion from Jeg3/CD1d cells is induced by the presence of decidual lymphocytes that include CD1d-restricted iNKT cells. Decidual lymphocytes were isolated from normal human decidual tissues collected at 7–9 gestational weeks. 10^5 decidual lymphocytes were cultured with Jeg3 or Jeg3/CD1d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

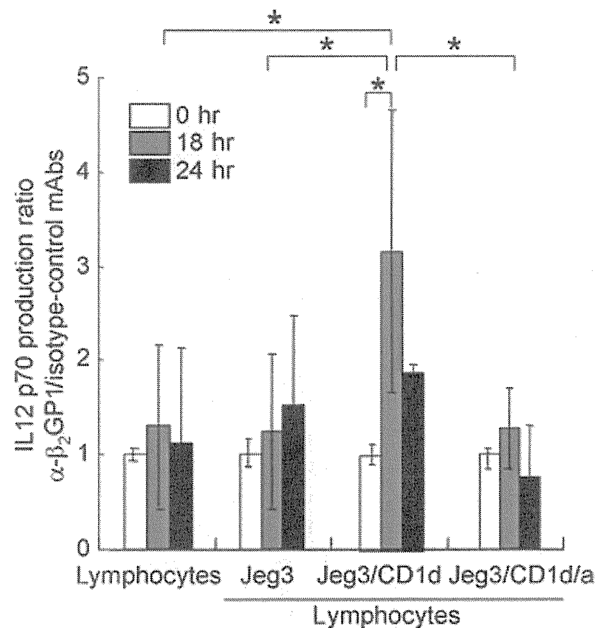

Fig. 7 Anti-β_2GP1 mAb enhances IL12 secretion during co-culture of Jeg3/CD1d cells and decidual lymphocytes. Decidual lymphocytes were co-cultured with Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells. Decidual lymphocytes were also cultured alone as an internal control. Cultured cells were exposed to anti-β_2GP1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

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

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

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

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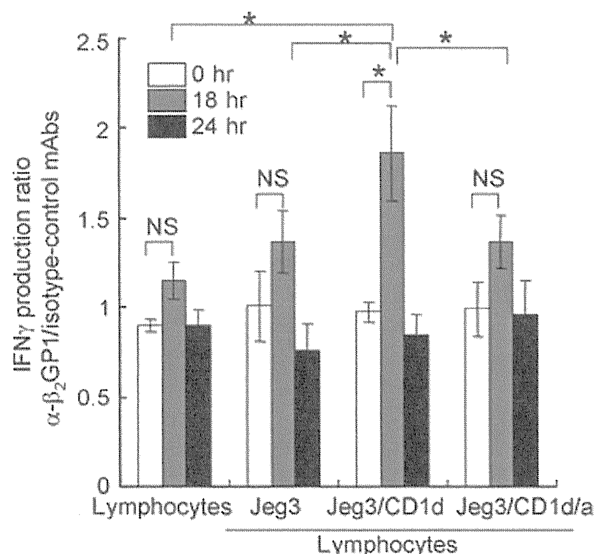

Fig. 8 Anti-β_2GP1 mAb enhances IFNγ secretion when added to co-cultures containing Jeg3/CD1d cells and decidual lymphocytes. IFNγ production in culture media collected for Fig. 7 was measured by ELISA and the production of IFNγ expressed as a ratio of anti-β_2GP1-exposed over control mAb-exposed specimens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

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

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

Discussion

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

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

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

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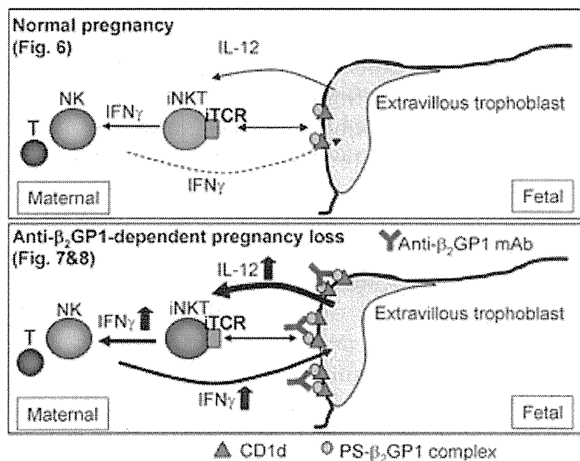

Fig. 9 Comparison of immune interactions at the human maternal–fetal interface during normal pregnancy with those proposed during anti-β_2GP1 mAb-related pregnancy loss. (Upper) In normal pregnancy, interaction of CD1d on the extravillous trophoblast cells (EVT) and maternal iNKT cells induces low level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

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

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

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

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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

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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

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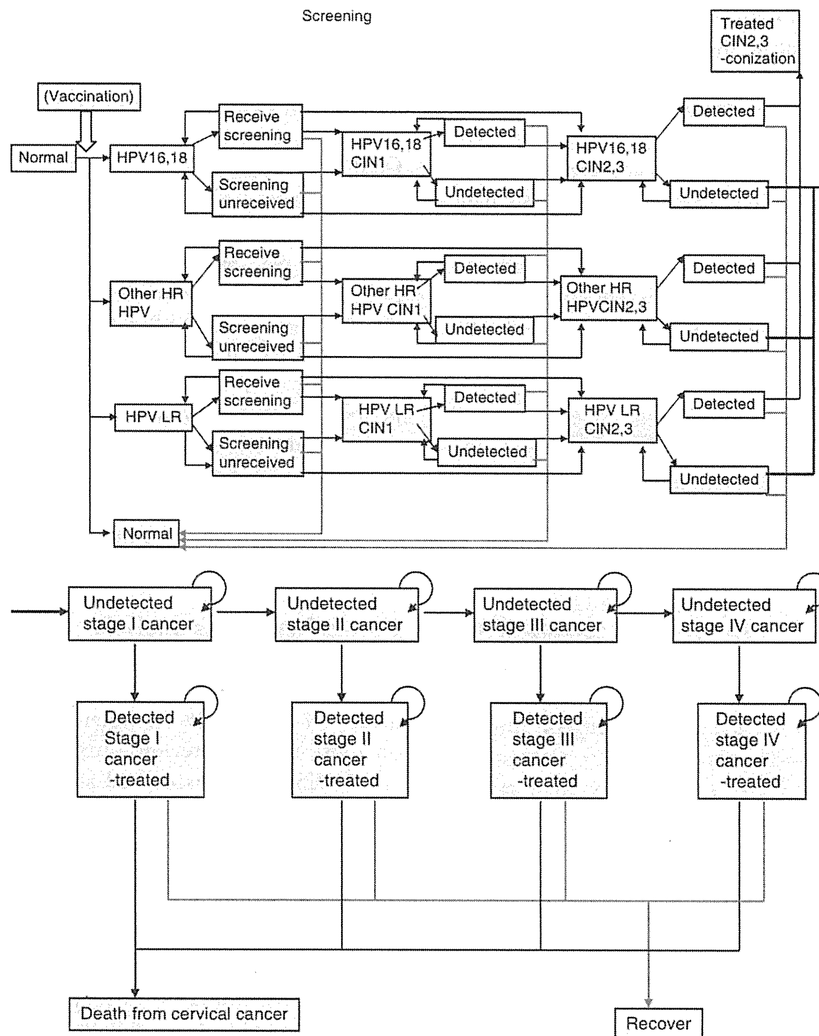

Figure 1. HPV natural history model.

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

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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

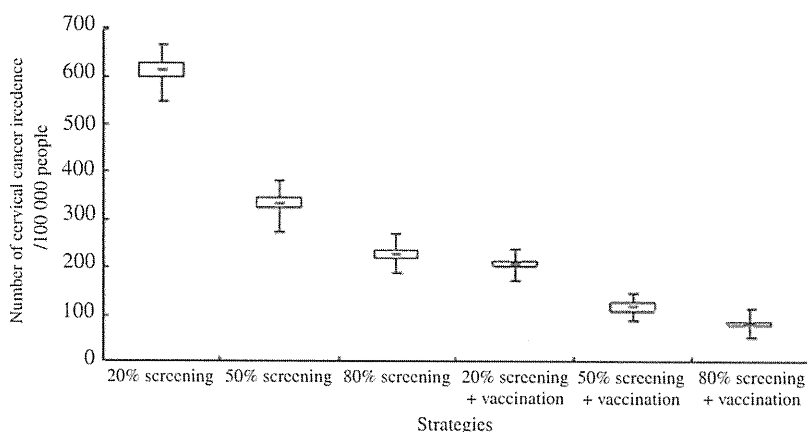

Figure 2. Lifetime risk of cancer for each strategy.

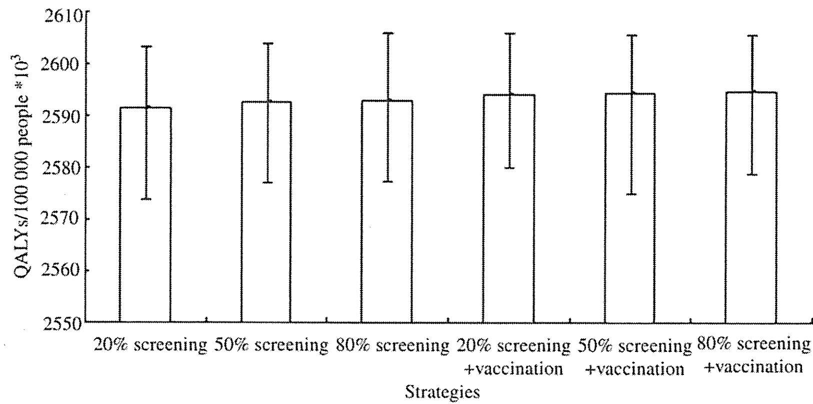

Figure 3. Total QALYs per 100,000 people for each strategy.

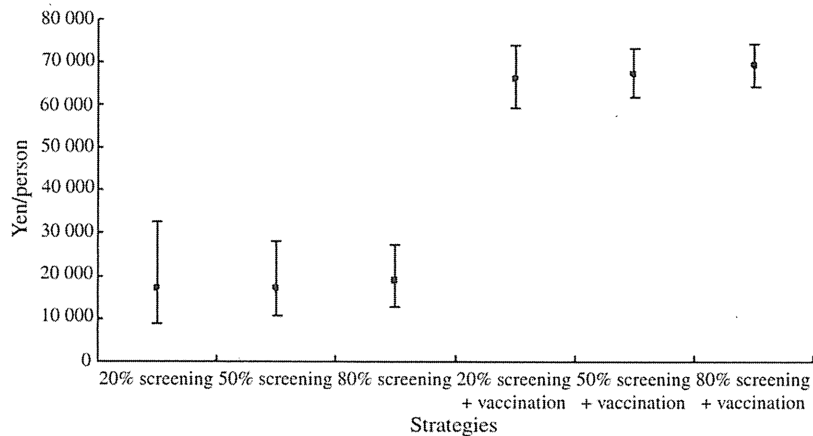

Figure 4. Cost per person for each strategy.

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.

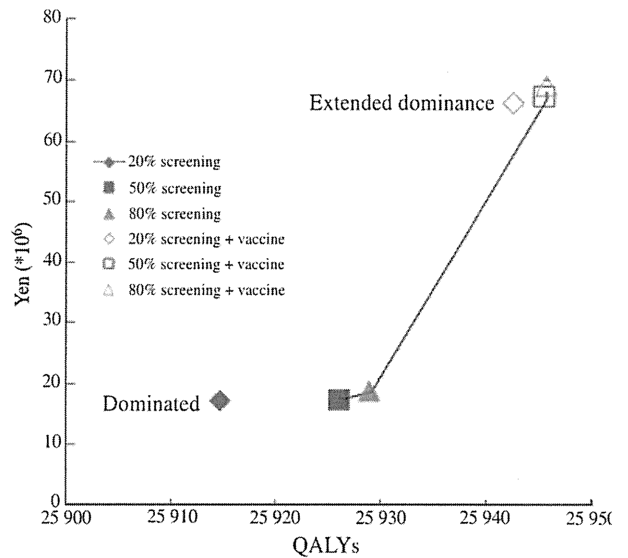

Figure 5. Cost and QALYs per 1000 people.

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

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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.

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The detection rate of HPV16 and 18 among women with cervical cancer in Japan is reported to be lower than that in other countries.[14] We used the latest age-dependent prevalence data, which consistently show that the younger population has a higher detection rate of HPV16 and 18 than the older population.[20] The prevention of cervical cancer in a young person shows larger QALYs gained than that of an older person because of the longer remaining life expectancy. Hence the effect of vaccine on cancer incidence or QALYs is not as low as might otherwise be expected.

The present study has several limitations. First, we assumed life-long lasting immunity acquired by the vaccine. The vaccine has only been recently introduced, and the latest evidence shows 7.3 years of efficacy and immunogenicity of the vaccine, which was derived from the population of the initial placebo-controlled study.[40] If additional vaccination is required to maintain immunity in the future, then programme costs are slightly underestimated. Second, there is no population-based survival data of women with cervical cancer by stages of FIGO. These data are essential when building a model. However, we managed to adopt and validate data from an existing Japanese regional cancer registry. Third, we did not incorporate the preferences of girls and their parents and the subsequent uptake of vaccine as a result of their preferences. Both effects and costs may be overestimated in that sense. Finally, we did not include the cost for campaigns to increase the coverage of screening and/or vaccination in this analysis, which may underestimate the programme costs but such a bias is minimal given the fact that the majority of costs is incurred by screening, vaccination and treatment interventions.

Vaccination for HPV is attracting considerable policy attention now as a strategy for cervical cancer prevention in Japan. Our analysis showed that increasing the rate of the current screening strategy would halve cancer incidence with a similar cost to the current screening strategy, though vaccination strategies may also be cost effective. We suggest further efforts to expand the current screening programme regardless of what support is provided for vaccination.

Some of the reasons why Pap smear coverage is so low in Japan relate to a lack of knowledge and from the fact that the financial support of the screening programme from the Ministry of Health, Labour and Welfare was discontinued because it was included in the general ones in 1998. Most cities, towns and villages decided to reduce the cost for the screening programme.[41–43] Free tickets for the Pap smear were provided under supplemental budgets for 2009. Distributing free tickets to a target population of certain ages showed a significant increase in the coverage rate by 2.8 times.[44] We need to continue endeavours to increase coverage by effective interventions such as providing free tickets and undertaking awareness campaigns. The involvement of gynaecologists in school education will also support the enhancement of knowledge about cervical cancer prevention and help to increase the coverage rate of screening as has been seen in other countries.[45,46]

Our analysis showed that introducing the HPV vaccination for all 11-year-old girls would reduce cervical cancer incidence to 33.9% with a net cost of only 49,000 yen per person (taking into account the social burden of cancer). Vaccinating all 11-year-old girls would cost 33.7 billion yen. Our analysis showed the cost-effectiveness of vaccination and that it would save future costs. It is important to give priority to policy which is evidence based medically and economically. If the prevalence of HPV infection is reduced as a result of universal vaccination, as our model predicts, then it may be possible to extend the interval between routine screens or to increase the age at which screening is first offered, as suggested in other cost-effectiveness studies.[34,47] The use of the HPV-DNA test in the screening programme is one choice that should be evaluated in the future.

In conclusion, the introduction of HPV vaccine in Japan is cost-effective as in other countries. It is more cost-effective to increase the coverage of the Pap smear along with the universal administration of HPV vaccine. Only by doing so, can the scarce healthcare resources be efficiently and effectively used to reduce the burden from cervical cancer in Japan.

Disclosure of interests

None of the authors have any conflicts of interest to declare.

Contribution to authorship

NY contributed to the study design of the current paper, model construction, data acquisition, data analysis and interpretation, drafting and revising the manuscript. RM contributed to the study design of the current paper, model construction, results interpretation and revising the manuscript. PJ contributed to the model construction, results interpretation and the critical review of the manuscript. YO contributed to the study design of the current paper. KK contributed to the model construction, data acquisition and interpretation of the results. KS and YT contributed to the study design of the current paper and interpretation of the results. All authors approved the final version of the manuscript.

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Characterization of Gut-Derived Intraepithelial Lymphocyte (IEL) Residing in Human Papillomavirus (HPV)-Infected Intraepithelial Neoplastic Lesions

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Problem
Mucosal T cells are the most likely direct effectors in host anti-human papillomavirus adaptive immunity and regression of cervical intraepithelial neoplasia (CIN) lesions. There are no studies addressing intraepithelial lymphocytes (IELs) in CIN lesions.

Method of study
Cervical lymphocytes were collected using cytobrushes from patients with CIN and analyzed by FACS analysis. Comparisons were made between populations of cervical T cells in CIN regressors and non-regressors.

Results
A median of 74% of cervical lymphocytes were $CD3^+$ T cells. Populations of integrin $\alpha E\beta 7^+$ IEL in CIN lesions varied markedly among patients (6–57%). Approximately half of integrin $\beta 7^+$ T cells were CD45RA-negative memory T cells. The number of integrin $\alpha E\beta 7^+$ cells among cervical T cells was significantly higher in CIN regressors when compared to non-regressors.

Conclusion
Higher cervical IEL numbers are associated with spontaneous regression of CIN. Accumulation of cervical integrin $\alpha E\beta 7^+$ IEL may be necessary for local adaptive effector functions.

Introduction

Lymphocytes involved in the mucosal immune system are found in the inductive sites of organized mucosa-associated lymphoid tissues (MALT) and in a variety of effector sites such as the mucosa of the intestine, respiratory tract, and genital tract.[1] The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin α4β7 and C-C chemokine receptor type 9 (CCR9). Lymphocyte-expressed integrin α4β7 and CCR9 bind to their natural ligands, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25 (TECK), respectively, which are expressed on the cell surface of

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endothelial cells in submucosal post-capillary venules.[1] In the intestine, mucosal dendritic cells (DCs) in gut-associated lymphoid tissues (GALT) regulate the expression of integrin α4β7 on activated effector and regulatory lymphocytes in a retinoic acid–dependent manner.[1–3] Mucosal T cells expressing integrin $\alpha4\beta7^+$ are known to circulate in peripheral blood from inductive sites and to home to the lamina propria (LP) at effector sites via α4β7-MAdCAM-1 and CCR9-CCL25 interactions.[4] Integrin $\alpha4\beta7^+$ T cells can differentiate into $\alpha E\beta7^+$ T cells upon exposure to TGF-β,[5] and the expression of integrin αEβ7 facilitates the retention of lymphocytes in the epithelium via interactions with E-cadherin.[4] Integrin αEβ7 is a specific marker of intraepithelial lymphocytes (IELs) residing in mucosal epithelia, and those cells expressing this antigen on their surface were initially educated in the gut.

The cervical mucosa is a very common site for pathogen invasion and is the primary transmission site for human papillomavirus (HPV), *Chlamydia trachomatis*, and human immune deficiency virus type 1 (HIV-1). A well-organized mucosal defense system in the cervical mucosa is critical to human health. Mucosal epithelial cells in the human cervix are active participants in such immunological protection.[6] However, the lymphocytes populating the cervical mucosal tissues, especially cervical IELs, have been poorly studied. Mucosal T cells in the murine genital tract express a large amount of integrin α4β7 on their cell surface,[7] and MAdCAM-1 is expressed on endothelial cells in the submucosa of murine fallopian tubes infected with *C. trachomatis*.[8] Several studies have demonstrated that human genital mucosa expresses MAdCAM-1 endogenously[9] and that GALT-derived integrin $\alpha4/E\beta7^+$ T cells home to the genital mucosa.[10,11] This T-cell homing and the expression of integrin αE increase in the presence of cervicitis and vaginitis.[10,11] Although integrin $\beta7^+$ mucosal T cells have been found in the cervical mucosa, a local inductive site (i.e., MALT) has never been demonstrated histologically.[11] We hypothesized that GALT may act as the inductive site for cervical IELs.

Human papillomavirus infection is a major cause of cervical cancer, and its precursor lesion, cervical intraepithelial neoplasia (CIN), develops in the epithelium. Natural history studies of CIN[12,13] show that most infections and CIN lesions resolve spontaneously but some persist and progress to cervical cancer. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions[14,15] suggest that cell-mediated immune response against HPV antigens is important in the control of HPV infection and progression to CIN. More controversial are the relative roles of systemic and local mucosal immune responses in the HPV pathogenesis. Trimble et al.[16] reported that naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN 2/3 lesions, but Nakagawa et al.[17] demonstrated a positive association between systemic cell-mediated immune responses to HPV E6 and HPV/CIN regression.

We studied the local mucosal cell–mediated immune response to HPV antigens by characterizing cervical mucosal immune cells collected non-invasively, using only a cytobrush. We confirmed that the collected $CD3^+$ cervical T cells were intraepithelial in origin (integrin $\alpha E\beta7^+$ IELs). Approximately half of the integrin $\beta7^+$ T cells were memory T cells. Finally, integrin $\beta7^+$ intraepithelial T cells increased significantly in the patients whose CIN lesions regressed spontaneously regardless of HPV genotype.

Materials and methods

Study Population

Cervical cell samples were collected using a cytobrush from 86 patients under observation after being diagnosed with CIN by colposcopically directed biopsy. All women gave written informed consent, and the Research Ethics Committee of the University of Tokyo approved all aspects of the study. Patients with known, symptomatic, or macroscopically visible vaginal inflammation or sexually transmitted infections were excluded from our study. Samples for HPV genotyping were collected at the first follow-up examination after diagnosis. Cervical lymphocytes were collected from non-menstruating patients at their latest follow-up visit. To study the potential association between cervical IEL characteristics and CIN progression, CIN patients with the regression of cervical cytology (cases) were matched with control patients who did not exhibit cytologic regression over the same time period (measured from initial detection of abnormal cytology). In this study, cytological regression was defined as normal cytology at two or more consecutive evaluations conducted at 3 to 4-month intervals. Thirteen patients were enrolled in the regression group, and the median

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follow-up duration was 27 (12–38) months. Thirteen pairs of follow-up time-matched patients with persistent cytological abnormalities were enrolled in the non-regression group, and the median follow-up time was 24 (12–40.5) months.

HPV Genotyping

DNA was extracted from cervical smear samples using the DNeasy Blood Mini Kit (Qiagen, Crawley, UK). HPV genotyping was performed using the PGMY-CHUV assay method.[18] Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and human leukocyte antigen-DQ (HLA-DQ) primer sets. Reverse blotting hybridization was performed. Heat-denatured PCR amplicons were hybridized to specific probes for 32 HPV genotypes and HLA-DQ reference samples. The virological background (HPV genotyping) of 86 patients in our study was shown in Table I. Here, HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs according to International Agency for Research on Cancer multicenter study.[19]

Table I Human Papillomavirus (HPV) Genotype Distribution

HPV type	Total numbers (%)
16	19 (18.4)
18	7 (6.8)
31	2 (1.9)
33	1 (1.0)
35	1 (1.0)
39	1 (1.0)
45	1 (1.0)
51	7 (6.8)
52	20 (19.4)
53	4 (3.9)
56	3 (2.9)
58	12 (11.7)
59	3 (2.9)
68	3 (2.9)
82	1 (1.0)
6	2 (1.9)
54	1 (1.0)
55	1 (1.0)
66	4 (3.9)
69	1 (1.0)
70	3 (2.9)
83	3 (2.9)
84	2 (1.9)
Total	103 (100)

Patients infected with multiple HPV types were included. Of 86 patients, 32 (37%) were infected with multiple types. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs.[19]

Collection and Processing of Cervical Specimens

Cervical cells were collected using a Digene cytobrush as described previously.[20] The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was placed in a 15-mL tube containing R10 media [RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B] and an anticoagulant (0.1 IU/mL of heparin and 8 nM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37°C for 15 min with shaking, the cytobrush was removed. The tube was centrifuged at $330 \times g$ for 4 min. The pellet was resuspended in 10 mL of 40% Percoll, layered onto 70% Percoll, and centrifuged at $480 \times g$ for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was >95%, as confirmed by trypan blue exclusion test, and fresh samples were immediately used for further analysis.

Immunolabeling and Flow Cytometry

Cervical immune cell preparations were immunolabeled, incubated on ice for 30 min, washed twice with FACS buffer (10% FCS, 1 mM EDTA, and 10 mM NaN_3) and fixed by adding paraformaldehyde in PBS to a final concentration of 1%.

The following fluorochrome-conjugated mouse monoclonal antibodies specific for human leukocyte surface antigens were used: a fluorescein isothiocyanate (FITC)-conjugated pan leukocyte marker (FITC-anti CD45), a B lymphocyte marker (FITC-anti CD19), a cytotoxic T-cell marker (FITC-anti CD8), a helper T-cell marker (FITC-anti CD4), an integrin β7 marker (FITC-anti integrin β7), a phycoerythrin (PE)-conjugated integrin α4 marker (PE-anti integrin α4), an integrin αE marker (PE-anti integrin αE), a C-C chemokine receptor type 9 marker (PE-anti CCR9), a marker for naïve cells (PE-anti CD45RA), a phycoerythrin cyanine 5 (PC5)-conjugated pan T lymphocyte marker (PC5-anti CD3), a natural killer cell marker (PC5-anti CD56), and an allophycocyanin (APC)-conjugated pan T lymphocyte marker (APC-anti CD3). Cell preparations were labeled in parallel with appropriate isotype control

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antibodies. Antibodies were purchased from eBioscience (San Diego, CA, USA) and Beckman Coulter (Brea, CA, USA). Data were acquired using three-color flow cytometry on FACSCalibar (Becton-Dickinson, Texarkana, TX, USA). The positions of lymphocytes and monocytes were determined on the forward scatter versus side scatter (SSC) profile. The positions of pan-lymphocytes and T lymphocytes were determined by CD45 and CD3 gating, respectively. As the percentage of B cells among cervical lymphocytes is known to be low (less than a few percentage) when compared to the 20% level seen in peripheral blood,[20] the presence of elevated $CD19^+$ B cells in cervical specimens would indicate contamination with peripheral blood. For our investigations, cervical samples with more than 3% B cells were excluded from analysis.

Statistical Analysis

Statistical analyses, including calculation of medians and interquartile ranges (IQRs), were performed using the commercial statistical software package JMP® (SAS, Cary, NC, USA). Wilcoxon rank sum test or Fisher's exact test was applied for matched paired comparisons. *P*-values ≤0.05 were considered significant.

Results

Purification of Cervical Leukocytes Collected from CIN Lesions

To characterize mucosal cellular immune responses in HPV-infected lesions, cervical samples, including exfoliated epithelial cells and cervical lymphocytes, were collected from CIN lesions positive for any HPV genotype using a cytobrush. Cervical samples were fractionated over a discontinuous Percoll density gradient to remove cervical epithelial cells, and the layer between Percoll and culture medium was collected. Cervical lymphocytes were identified among isolated cells using standard SSC and CD45 gating (Fig. 1). Approximately 10^4–10^5 $CD45^+$ cells were isolated from patients' cervices. $CD45^+$ cells primarily consisted of lymphocytes (Fig. 1, circle) and granulocytes (Fig. 1, square). A minority of the cells included in the square in Fig. 1 were monocytes (data not shown). Two representative cases are provided in Fig. 1: the upper panel represents a patient with numerous granulocytes and a relatively small population of $CD45^+$ lymphocytes (10%), whereas the lower panel represents a patient with few granulocytes and a high number of lymphocytes (30%).

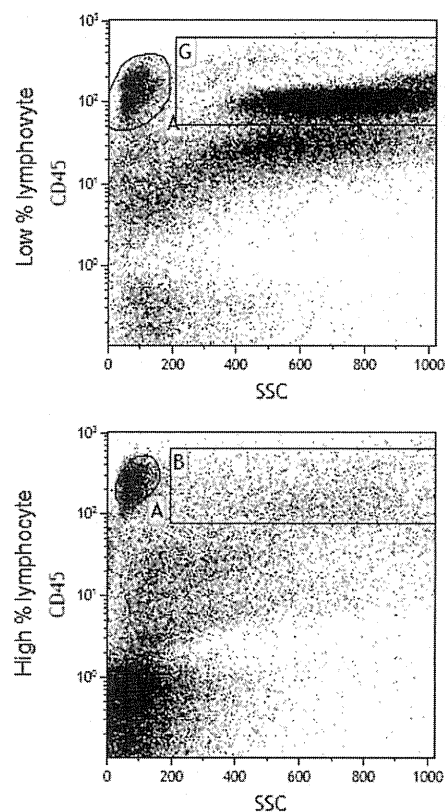

Fig. 1 Flow cytometric analysis of cervical mucosal cells using CD45/SSC gating. Processed cervical specimens were analyzed by flow cytometry and CD45/SSC gating. $CD45^+$ cervical leukocytes are comprised of lymphocytes (circle) and granulocytes/monocytes (square). Upper and lower panels were representative of patients with low (about 10%) and high (about 30%) numbers of lymphocytes among their $CD45^+$ cervical leukocytes, respectively. The absolute number of isolated cervical lymphocytes remained relatively constant among study subjects.

Characterization of Cervical T Cells in CIN Lesions

The majority of cervical lymphocytes isolated from CIN lesions were $CD3^+$ T cells [median 74% (IQR: 59–82)]. $CD19^+$ B cells were rarely found [median 0.45% (IQR: 0.04–1.40)]. In Fig. 2, CD3-gated cervical T cells were characterized by flow cytometry, and each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively. A median of 54%

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