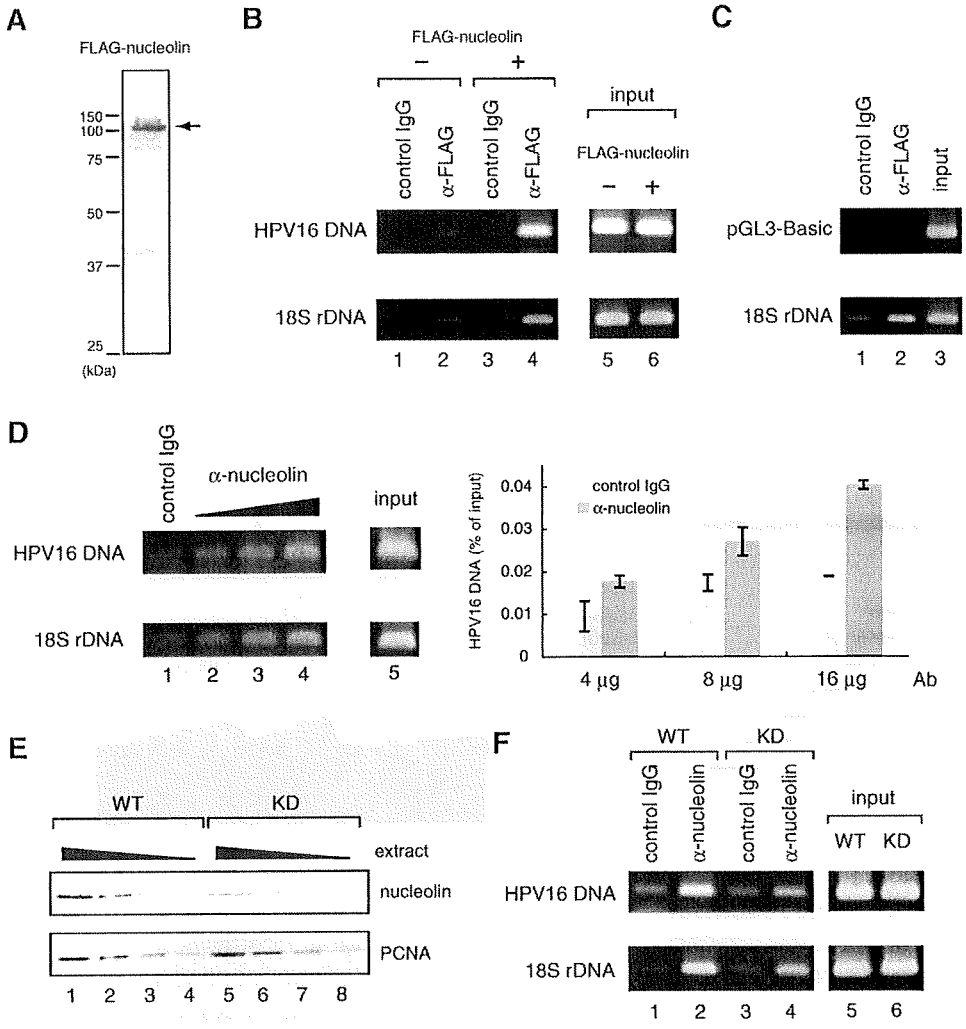

Fig. 3. Binding of nucleolin to HPV16 DNA in HeLa cells. (A) Western blot analysis of exogenous FLAG-nucleolin in HeLa cells using anti-FLAG antibody. (B) Chromatin immunoprecipitation analysis to detect binding of FLAG-nucleolin to HPV16 DNA in HeLa cells transfected with pGL3-P_{670}, which contains the HPV16 genome sequence from nt 7003 to 868, together with the FLAG-nucleolin expression plasmid or its backbone plasmid. Cross-linked FLAG-nucleolin/DNA complexes were immunoprecipitated with anti-FLAG antibody (lanes 2 and 4) or control mouse IgG (lanes 1 and 3), and the immunoprecipitated DNA was purified and subjected to PCR for the HPV16 DNA (nt 501–670) or 18S rDNA. The PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide stain. Part (0.3%) of the input chromatin was analyzed. (C) ChIP analysis using HeLa cells transfected with pGL3-Basic and the FLAG-nucleolin expression plasmid. ChIP was performed with anti-FLAG antibody (lane 2) or control IgG (lane 1), followed by PCR for the pGL3-Basic sequence or 18S rDNA. (D) ChIP analysis to detect binding of endogenous nucleolin to the HPV16 DNA in HeLa cells transfected with pGL3-P_{670} alone. Anti-nucleolin antibody (Santa Cruz) was used to recover DNA/nucleolin complexes. The increasing amounts of antibodies (lanes 1 and 2, 4 μg; lane 3, 8 μg; lane 4, 16 μg) were used for ChIP. The amounts of immunoprecipitated HPV16 DNA were quantified by real-time PCR and shown as a percentage of the input HPV16 DNA in the right panel. Results are presented as means ± standard errors of two independent experiments. (E) Western blot analysis using total cell extracts from wild-type (WT) and nucleolin-knockdown (KD) HeLa cells. Twofold serially diluted extracts (lanes from 1 to 4 for WT cells; lanes from 5 to 8 for KD cells) were subjected to Western blotting with anti-nucleolin (Active Motif) or anti-PCNA antibodies. (F) ChIP analysis using wild-type and nucleolin-knockdown HeLa cells. Cross-linked nucleolin/DNA complexes from WT (lanes 1 and 2) and KD cells (lanes 3 and 4) were immunoprecipitated with anti-nucleolin antibody (Abcam) (lanes 2 and 4) or control mouse IgG (lanes 1 and 3), and the immunoprecipitated DNA was subjected to PCR for the HPV16 DNA (upper panel) or 18S rDNA (lower panel).

Discussion

Here we report the preferential binding of nucleolin to the HPV16 genomic region from nt 531 to 780. This genomic region is a "hot spot" for interactions with many cellular transcription factors that regulate the late promoter P_{670}. CCAAT displacement protein (CDP) and YY1 associate with this region to suppress the basal transcription from P_{670} [16–18], whereas multiple bindings of hSkn-1a and CCAAT/enhancer-binding protein β (C/EBPβ) to the same region relieved the repression by CDP and YY1, leading to activation of P_{670} [11,19]. Nucleolin bound to the HPV16 genomic region from nt 591 to 620 and recognized the sequence motif 5′-TTGCXXXCAXA-3′ from nt 604 to 614. This sequence partially overlaps with a binding site for C/EBPβ [11], which suggests functional competition between C/EBPβ and nucleolin for P_{670} regulation. However, contrary to the previous report showing an enhancing effect of nucleolin on the HPV18 early promoter [14], neither expression of FLAG-nucleolin nor knockdown of endogenous nucleolin by RNAi in HeLa cells had obvious effects on the P_{670} activity in a transient reporter assay (Sato et al., unpublished observation), questioning nucleolin's role in the HPV transcription.

Since nucleolin associated with the HPV16 genome in W12 cells, the nucleolin binding to the HPV16 genome likely occurs in the context of natural infection. With regard to maintenance of the viral genome, it is worth noting that a plasmid containing an HPV16 genomic fragment from the E6 to E7 region can be stably

Please cite this article in press as: H. Sato et al., Identification of nucleolin as a protein that binds to human papillomavirus type 16 DNA, Biochem. Biophys. Res. Commun. (2009), doi:10.1016/j.bbrc.2009.07.055

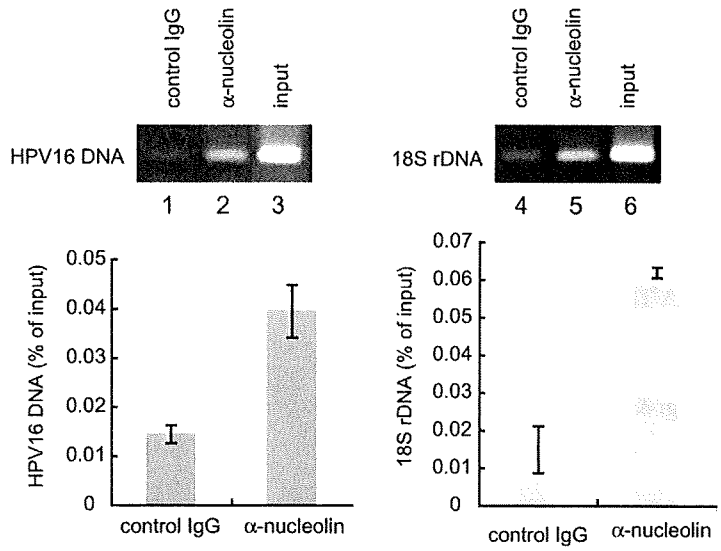

Fig. 4. Binding of nucleolin to HPV16 genome in W12 cells. ChIP analysis using W12 cervical neoplasia cells to detect binding of endogenous nucleolin to the HPV16 genome or rDNA. Cross-linked nucleolin/DNA complexes were immunoprecipitated with anti-nucleolin antibody (Abcam) (lanes 2 and 5) or control mouse IgG (lanes 1 and 4), and the immunoprecipitated DNA was subjected to PCR for the HPV16 DNA (nt 501–670) (lanes 1 and 2) or 18S rDNA (lanes 4 and 5). Part (0.3%) of the input chromatin was analyzed (lanes 3 and 6). The amounts of immunoprecipitated DNA were quantified by real-time PCR and shown as a percentage of the input DNA. Results are presented as means ± standard errors of two independent experiments.

maintained in human cells in the absence of the viral E1 and E2 proteins [20]. This finding indicates a genome maintenance mode that is completely dependent on host proteins (independent of virally encoded proteins). Given that the nucleolin-binding site located from nt 604 to 614 lies in the E7 region, nucleolin may facilitate E1/E2-independent maintenance of the HPV genome. Detailed analysis of subcellular localization of nucleolin has demonstrated that nucleolin becomes localized at the chromosome periphery during mitosis in HeLa cells [10]. Thus, nucleolin might be involved in tethering the HPV genome to host chromosomes, which prevents the HPV genome from diffusing into cytoplasm during mitosis.

Lastly, nucleolin has been reported to play roles in the life cycle of other human viruses. Nucleolin interacts with nonstructural protein 5B of hepatitis C virus (HCV) and this interaction seems to be required for efficient replication of HCV [13]. Nucleolin localizes to the viral replication compartments of herpes simplex virus type 1 (HSV-1) in the nucleus during productive infection and the knockdown of nucleolin by RNAi inhibits HSV-1 replication [21]. Thus, it will be of particular interest to investigate the exact function of nucleolin in the HPV life cycle in future studies.

Acknowledgment

We thank Dr. Paul Lambert for providing us with the W12 cell line.

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Please cite this article in press as: H. Sato et al., Identification of nucleolin as a protein that binds to human papillomavirus type 16 DNA, Biochem. Biophys. Res. Commun. (2009), doi:10.1016/j.bbrc.2009.07.055

Review Article

Indian J Med Res 130, September 2009, pp 341-347

Human papillomavirus vaccines: current issues & future

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Received February 16, 2009

Cervical cancer is the leading cause of cancer mortality among women in worldwide. Some 99 per cent of cervical cancer cases are linked to genital infection with human papillomaviruses (HPVs) comprised of approximately 15 oncogenic genital HPV types. Most HPV infections resolve spontaneously. But, the remainder persist and may then progress to cervical cancer in some women. In high-resource countries, the best way to prevent cervical cancer is to implement organised gynaecological screening programs with appropriate treatment of the detected pre-cancerous lesions. However, in developing countries, this method is not practicable because of cost and complexity of proper screening. Vaccines against HPV infections hold promise to reduce incidence of cervical cancer cost-effectively. Two Prophylactic HPV vaccines have been thus far developed: Gardasil®, a quadrivalent vaccine targeting HPV-6, -11, -16 and -18) and Cervarix®, a bivalent vaccine which targets HPV-16 and -18. Both vaccines contain L1 virus-like particles (VLPs) derived from HPV-16 and -18 which are most frequently associated with cervical cancer. The L1-VLP vaccines are HPV type-specific and therefore can effectively prevent infection of a HPV type in question alone. Therefore, the L1-VLP vaccines are hoped to be multivalent for 15 oncogenic HPV types, which comes at a price. Otherwise, costly cytologic screening for cervical cancer is still necessary. The current HPV vaccines thus may not be ultimate strategy and study on new HPV vaccines is needed. Broad-spectrum prophylactic vaccines against all oncogenic HPV types and therapeutic vaccines for clearance of HPV-related cervical lesion are being developed.

Key words Cervical cancer - human papillomavirus (HPV) - HPV vaccines

Epidemiology of HPV infection

At present, there are about 100 identified genotypes (types) of human papillomavirus (HPV), of which about 40 are genital HPV types that invade the genital organs such as the uterine cervix, vaginal wall, vulva, and penis. Genital HPV types are classified into high-risk types commonly associated with cervical cancer and low-risk types known causative pathogens of condyloma acuminatum. This classification varies among researchers, but, in general, types 16/18/31 /33/35/39/45/51/52/56/58/66/68 are classified as high-risk and 6/11/40/42/43/44/54/61/72 as low-risk types[1]. Interestingly, the HPV type distribution varies depending on the discrete stage of cervical neoplasia (Fig. 1).

The HPV-DNA detection rate in the genital organs of healthy adult females varies between advanced and developing countries, being approximately 20-40 per cent collectively[2,3]. In Japan, the HPV-positive rate in pregnant women aged 20-29 yr has been reported to be 20-30 per cent similar to, or higher than in the same age group in the US[4]. The World Health Organization

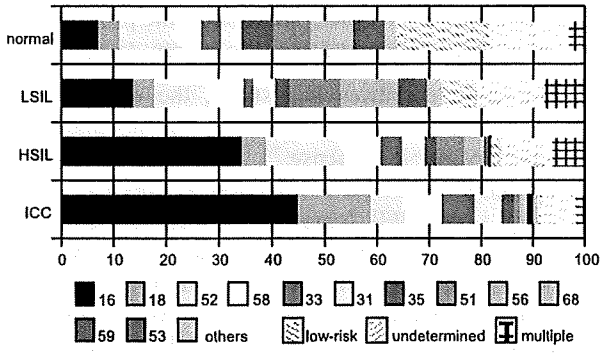

Fig. 1. HPV type distribution in cervical neoplasia in Japan[18]. HPV16 and 18 are the most common types in invasive cervical cancer (ICC) while more than 40 per cent of the invasive cancer is associated with the other types in Japan. HPV52 is the most common type in female with normal cytology in Japan[18].

(WHO) estimated an annual increase of 3 hundred million in the number of HPV carriers in the world[5,6]. Overall HPV prevalence in 157,879 women with normal cervical cytology was estimated to be 10.4 per cent[6]. In the US, epidemiological data show HPV infection at least once in life in 3 out of every 4 females[3]. Thus, HPV infection is a common disease affecting any female but not an event those in particular populations. High sexual activity has been reported to increase the risk of HPV infection[7]; in some women.

Risk factors for the progression of cervical neoplastic diseases

The incidence of cervical epithelial dysplasia (corresponding to squamous intraepithelial lesion: SIL) is about 1 per 10 females with HPV infection[8]. The incidence of high-SIL (corresponding to cervical intraepithelial neoplasia 2 and 3: CIN2 and CIN3, respectively) is about 3 per 10 females with low-SIL, and that of CIN3 is about 1-2 per 10 females with low-SIL[9]. Since therapeutic interventions are performed for CIN3, the actual incidence of cervical cancer is about 1 per 600 females with HPV infection. Without treatment, the incidence of the progression of CIN3 to cervical cancer is about 30 per cent[10]. Therefore, the incidence of the spontaneous development of cervical cancer is about 1 per 200-300 females with HPV infection.

Factors associated with progression to cervical cancer in females with HPV infection have been extensively studied[1]. Many prospective studies have identified persistent HPV infection as the most important risk factor, and also showed that the persistent infection tends to occur in high-risk type HPV. Persistent HPV infection generally involves persistent virus proliferation, as verified by the detection of virus DNA from cervical exfoliated cells. Chronic virus proliferation induces the active proliferation/ differentiation of infected epithelial cells, and some infected cells incidentally immortalize, which is the first step of carcinogenesis[1].

On the other hand, transient infection involves short-term virus proliferation followed by long-term latent presence of low copies of the viral genome in the basal cells of the genital epithelium. A fate of HPV infection leading to transient, but not persistent, is determined by cellular immunocompetence against HPV. It is unlikely that transient infection progresses to cervical cancer[1].

Prophylactic vaccines

Development of the current L1-VLP vaccines

HPV is the causative virus (requirement) for genital cancers with cervical cancer being most prevalent. Thus, theoretically, if HPV infection could be completely eradicated, most of genital cancers could be prevented. The study of HPV vaccines began about 10 years ago. In 2002, Koutsky *et al* were the first to show the clinical prophylactic effects of an HPV vaccine[11]. Merck in the US and Glaxo Smith Kline (GSK) in Europe launched full-scale development of prophylactic vaccines against HPV, and their vaccines were approved and commercially available a few years ago. The vaccine antigens of the two companies are virus-like particles (VLP) produced using HPV type16 L1 protein overexpressed in yeasts or insect cells. These particles externally have a 3-dimensional structure similar to that of virus particles, but have no contents, and, therefore, are not infective. The vaccine reported by Koutsky *et al*[11] also uses HPV16L1-VLP as an antigen.

However, the main problem of the L1-VLP vaccine is its negligible prophylactic effects on other HPV types[12]. Therefore, GSK and Merck developed cocktail vaccines composed of L1-VLPs corresponding to HPV types as targets. The vaccine developed by Merck is a tetravalent vaccine against types 6, 11, 16, and 18 (Gardasil®)[13] and that developed by GSK was a bivalent vaccine against types 16 and 18 (Cervarix®)[15]. A follow-up after inoculation with the quadrivalent vaccine showed the prevention of persistent infection with all 4 HPV types in 96 per cent[14]. Though the antibody titers have been maintained for 4-5 years[13-15], whether the antibody titers can be maintained for longer periods is unknown.

Clinical trials led by the two companies are ongoing in Japan and elsewhere.

Issues regarding the currently prevailing L1-VLP vaccines

The current HPV vaccines developed by GSK and Merck are used for uninfected females to prevent HPV infection/spread. For mass prophylactic vaccination in uninfected females, vaccination should be performed at the age of about 10 years before sexual activity begins. A recent phase III clinical study (FUTURE 1 & 2) in which females aged about 20 years were randomly inoculated with Gardasil® revealed prophylactic effects on the development of CIN2-3 associated with HPV types 16 and 18 in more than 98 per cent of females who completed the vaccination protocol[16,17]. However, prophylactic effects were observed in only 13-22 per cent of females inoculated just once or twice or by intention-to-treat analysis including prophylactic effects on other HPV types[16,17].

At present, antibody titers induced by L1-VLP vaccines are confirmed to be maintained for only 5 yr. There is no guarantee that the prophylactic effects of the vaccine inoculated at the age of 10 yr will be maintained, beyond the sexual activity period. Even if the prophylactic effects of the current HPV vaccines continue for life, only cases of cervical cancer due to HPV types 16 and 18, which constitute less than 60 per cent of all invasive cervical cancer cases in Japan[18], can be prevented (Fig. 1). Indeed, the HPV type distribution in cervical cancer varies depending on regions in the world[19]. HPV16 and 18-associated cervical cancer is more than 70 per cent in North America, Europe and Australia, about 65 per cent in Africa, about 60 per cent in South and Central America, and less than 60 per cent in Asia including Japan[18,19]. Therefore, females who undergo vaccination and receive the current vaccine may have a risk for the development of cervical cancer and thereby need not undergo cervical cancer screening. Providing such information to females undergoing this vaccination is the most important for the introduction of the current HPV vaccines. A single dose of the present HPV vaccines costs about 100 USD. There is need for reduction of this high cost. In addition, the L1-VLP vaccines are highly protective against infection corresponding to the papillomavirus type used to derive the immunogen, but are ineffective against all but the most closely related HPV types. Therefore, the L1-VLP vaccines should be ultimately multivalent for 15 oncogenic HPV types. This makes the prophylactic vaccine more expensive than the current vaccines.

In some countries and states, the current HPV vaccines are distributed for free, or inoculation is covered by pubic expenses[20]. However, considering the progression of HPV infection to cervical cancer in only 1 per 300 females, vaccines effecting the prevention of only limited types, the relatively wide-spread cancer screening, and the high cost of such vaccines, it mandatory mass preventive inoculation with the current HPV vaccines is of value in developed country such as Japan may not be feasible. In addition, the current HPV vaccines targeting only HPV types 16 and 18 do not enable the omission of cancer screening, and vaccination at public expenses has no advantage in terms of medical economics. In Japan, voluntary inoculation during the sexual activity period should be performed first at the expense of each woman. The mass prevention employing the current HPV vaccines is a matter of debate.

Second generation HPV prophylactic vaccines

The main problem regarding the current L1-VLP vaccines is the induction of type-specific immunity. To overcome this, broad-spectrum vaccines that are also effective for the prevention of high-risk type HPV infection are under development. L2 as the other structural proteins of virus particles contains many conserved regions among all HPV types (Fig. 2). We

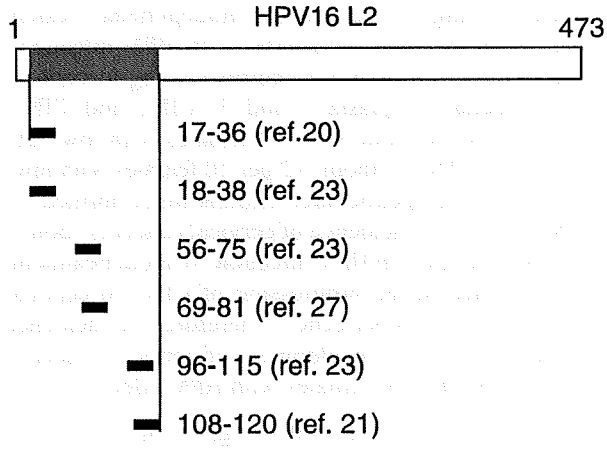

Fig. 2. Broad-spectrum neutralization epitopes of HPV16 L2 capsid protein. Many studies reveals linear epitope cross-neutralizing infection with many HPV types in L2 capsid protein. Each epitope includes amino acid conserved regions between genital HPV types and has potential of neutralization of HPV infection. These are candidates for type-common prophylactic vaccines to HPV[21,23,26,27].

and Kondo *et al* have sought a way to develop novel vaccines using partial regions of L2 containing type-common neutralization epitope[21,22]. Recently, Kondo *et al* identified a vaccine candidate for the prevention of all types by developing newly type-common neutralization epitopes of L2 and optimizing the regions[23]. Roden *et al.* also studied the type-common neutralization epitope of L2[24-26]. They devised strategies to use the entire L2 for vaccines, and their joint study with Christensen *et al*[20]. confirmed its suppressive effects on infection with a broad spectrum of HPV types in animal experiments[25]. Furthermore, they discovered a new region (17-36 amino acid of HPV16 L2) of L2 which contains broad-spectrum neutralization epitopes[26]. It is certain that L2 will be a vaccine antigen candidate for common-type vaccines for the prevention of HPV infection.

The problem of L2 is its lower antigenicity than that of L1-VLP[22]. To apply L2 to humans, there are various problems such as the incidence of non-responders to the vaccine and the necessity for adjuvants. Several groups have recently revealed that chimeric VLP in which the cross-neutralization epitope of L2 inserted induce cross-neutralizing antibodies more effectively[27,28]. If high-risk type HPV infection can be suppressed using L2, the benefits of mass prevention by prophylactic HPV vaccine should be increased.

Other vaccine strategies for cervical cancer

Vaccine and cancer prevention strategies for cervical cancer depend on the medical/economic situations of each country. In low-resource settings, prophylactic vaccines against HPV infections have clearly the potential to reduce incidence of cervical cancer cost-effectively. By contrast, in developed countries, where precursor lesions of cervical cancer can be detected early based on well-established cancer screening program, the following diverse vaccine strategies warrant consideration (Fig. 3): (*i*) vaccines for the prevention of infection in uninfected females, (*ii*) vaccines for the reduction of viral load at the cervical mucosa in females with low-SIL and prevention its progression, (*iii*) vaccines for treatment in females with high-SIL, and (*iv*) immunotherapy for cervical cancer. The current HPV vaccines are those for the prevention of infection described in (*i*). On the other hand, (*iii*) and (*iv*) are considered to be therapeutic vaccines used for females with disease, and many clinical studies on such vaccines have been performed worldwide[29]. However, none of the vaccines exhibited statistically significant clinical effects with

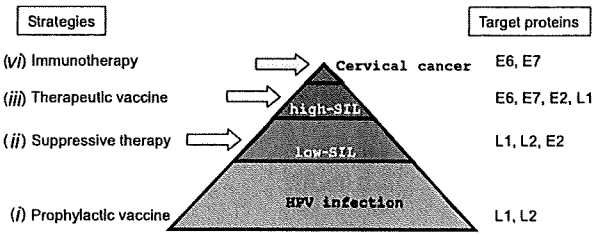

Fig. 3. Possible strategies utilizing immunological responses to HPV proteins for HPV-associated lesion and the target viral proteins for each strategy. (*i*) vaccines for the prevention of infection in uninfected females, (*ii*) vaccines for the reduction of viral load at the cervical mucosa in females with low-SIL to prevent from progression, (*iii*) vaccines for treatment in females with high-SIL, and (*iv*) immunotherapy for cervical cancer.

adequate cellular immunological responses induced by the vaccines. Since prophylactic vaccines such as the current HPV vaccines are preceding, the development of the latter seems to be delayed at present.

Possible suppressive therapy for cervical neoplasia

Long-term effects of the current HPV vaccines on HPV infection are still unclear. Clinical studies were already initiated by inoculating females aged about 20 yr with this HPV vaccine irrespective of the presence/absence of HPV infection. A recent study revealed that the current HPV vaccines tended to protect women who had already oncogenic HPV infection as well as cytological abnormalities from progression to high-grade CIN at 15 months follow-up[30]. We reported that HPV16-associated CIN1-2 tends to regress at 24 months follow-up in patients positive for serum high-titer neutralizing antibodies to HPV16[31]. Both evidences were not based on long-term follow-up. The current HPV vaccines are known to have a marked ability to induce neutralizing antibodies. Given these considerations, current HPV vaccines are likely to eliminate persistent HPV infection and subsequent malignant transformation. This raises the expectation that the vaccines can work so as to suppress HPV infection as described in 2). The results of further clinical studies are awaited.

Therapeutic vaccines

Because of limitations of the current HPV vaccines as mentioned above, necessity of therapeutic vaccines for the treatment of HPV-associated lesions is still in demand even after the prophylactic vaccine program are implemented in the world[29]. Development of the HPV therapeutic vaccines has been performed for the

Table. Clinical trials of therapeutic vaccine for HPV-associated cervical lesion

Trial phase	Target proteins	Vaccine vectors	Inoculation	Target HPVs
Ph-I/II[34]	L1, E7	Chimera-VLP	sc	16
Ph-II[33]	E7	Hsp (SGN-00101)	sc	16
Ph-II[35]	E6, E7	Vaccinia virus (TA-HPV)	sc	16, 18
Ph-II[36]	L2, E6, E7	Fusion protein L2E6E7 (TA-CIN)	im	16, 18
Ph-II[37]	BPV E2	Vaccinia virus (MVA-E2)	intrauteral	all
Ph-III[38]	E6, E7	Plasmid vaccine (ZYC101a)	im	16, 18

sc, subcutaneous injection; im, intramuscular injection; BPV, bovine papillomavirus

last two decades. The following vaccines have been well evaluated in clinical studies (Table).

1. SGN-00101 (sc) is a fusion protein consisting of heat shock protein (Hsp) of *Mycobacterium bovis* and HPV type 16 E7. The Ph-II study looking at effect of SGN-00101 in cases with CIN3 revealed histological CR in 13 (22.5%) of 58 cases, although immunological responses was not determined[32]. Another Ph-II study in cases with CIN showed 7 (35%) of 20 patients. In 5 of the 7 cases, the induction of CTL against HPV16E7 in peripheral monocytes was shown[33].

2. L1VLP-E7 (sc) is a vaccine using chimera particles composed of HPV type 16 L1-VLP and E7. In the Ph-I/II study in CIN2-3 cases, histological PR was shown in 39 per cent of vaccine recipients compared with 25 per cent of placebo recipients although there was no significant difference[34]. The clinical efficacy was coupled with cellular immune responses in some cases.

3. TA-HPV (im) is a recombinant vaccinia virus expressing HPV16/18 E6 and E7. The Ph-II study of TA-HPV in VIN cases revealed PR was shown in 8 of 13 cases and reduction of viral load was also shown in 6 of 8 lesion responders. The responders showed increase of lesion-infiltrating CD4 and 8-positive cells[35].

4. TA-CIN (im) is a fusion protein consisting of E6, E7 and L2 of HPV types 16 and 18. The Ph-II study in VIN cases revealed that CR or PR was shown in only 6 of 29 cases. CTL against E6/E7 was induced in 4 of 29 cases[36]. The correlation between clinical efficacy and cellular immune responses to the vaccine are unclear.

5. MVA-E2 (TGA4001) (intrauteral) is also a recombinant vaccinia virus expressing bovine papilloma virus (BPV) E2. The Ph-II study in cases with CIN2-3 confirmed antibody responses in serum, CTL induction in peripheral blood, and the regression of CIN in some cases (19/34 cases). There was no significant clinical efficacy[37].

6. ZYC-101a (im) is a DNA vaccine synthesized from some proteins containing CTL epitopes against E6 and E7 of HPV types 16 and 18. The Ph-III test was performed in subjects with CIN2-3. CR or PR was observed in 41 per cent in the vaccination group and 27 per cent in the placebo group, with no significant difference. When the cases were limited to those aged ≤ 25 yr, the percentage showing CR or PR was significantly higher in the vaccination (72%) than in the placebo (23%) group. However, no correlation between CTL induction against E6/E7 and clinical effects was shown[38].

Thus, there are no therapeutic HPV vaccines so far with apparent clinical efficacy based on enhanced cellular immune responses induced by vaccines. The current therapeutic vaccines elicit systemic cellular immunity by intramuscular or subcutaneous injection and thereby the clinical trials have shown cellular immune responses to the vaccines in peripheral monocyte, but not mucosal immunity at cervical mucosa.

We consider that CTL induction in the cervical mucosa is indispensable for treating cervical mucosal lesions such as CIN. In addition, vaccination is an effective method in the induction of mucosal immunity. Therefore, we have attempted induction of mucosal T cell responses by stimulating intestinal mucosal immunity through mucosal administration, particularly oral administration. Bermudez-Humaran *et al*[39]. produced gene-recombinant type lactic acid-expressing HPV16E7 and IL-12 from live lactobacillus, and evaluated the induction of CTL activity following its nasal or oral administration as a live vaccine in an experiment using mice, and also its preventive and

reductive effects in a tumor challenge test . They also found more marked mucosal induction after nasal than oral administration and a more effective induction of immunity using *Lactobacillus plantarum* than *Lactococcus lactis*[40]. No information on clinical studies of this vaccine is available. We have worked with a lactobacillus HPV vaccine using the *Lactobaccilus casei* strain showing of inflammatory immune responses. We noted marked induction of mucosal T cells possessing CTL activity to HPV E7 at intestinal mucosa after its oral administration of *Lactobaccilus casei* expressing HPV16 E7 to mice (Kawana *et al*, unpublished data). Further studies are necessary to get a detailed picture of this approach.

Summary

The usefulness of the current HPV vaccines cannot be underestimated. These vaccines are a valuable step toward the control of cervical cancer. The mass prevention strategy by use of the current HPV vaccine is ongoing in many countries. However, a conclusion cannot be drawn until the results of large-scale clinical studies in progress and long-term follow-up data are available. In addition, the development of the next generation HPV vaccines is also essential.

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ORIGINAL ARTICLE

Expression of Autotaxin, an Ectoenzyme that Produces Lysophosphatidic Acid, in Human Placenta

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Keywords
Autotaxin, lysophosphatidic acid, lysophospholipase D, placenta, pregnancy, trophoblast

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Submitted March 12, 2009;
accepted May 1, 2009.

Citation
Iwasawa Y, Fujii T, Nagamatsu T, Kawana K, Okudaira S, Miura S, Matsumoto J, Tomio A, Hyodo H, Yamashita T, Oda K, Kozuma S, Aoki J, Yatomi Y, Taketani Y. Expression of autotaxin, an ectoenzyme that produces lysophosphatidic acid, in human placenta. Am J Reprod Immunol 2009; 62: 90–95

doi:10.1111/j.1600-0897.2009.00715.x

Problem
Lysophosphatidic acid (LPA) is a bioactive lipid mediator and thought to play an important role in pregnancy. Plasma LPA is produced by autotaxin (ATX), and ATX activity in plasma increases during pregnancy paralleled with gestational weeks and decreases to near the non-pregnant level soon after delivery. However, the source of increased ATX during pregnancy is still uncertain. We hypothesized that the source of increased ATX might be placenta.

Method of study
We investigated the protein and mRNA expression of ATX in human placenta using immunohistochemistry and RT-PCR, respectively.

Results
At all 3 gestational trimesters, immunohistochemical staining for placenta tissues revealed the most marked positive staining of ATX protein in trophoblasts. Real-time PCR revealed that mRNA amounts of ATX in placenta tissues paralleled with gestational weeks, i.e. ATX level in plasma.

Conclusion
These findings suggest that trophoblasts might produce ATX and its bioactive resultant substance, LPA, paralleled with gestational weeks.

Introduction

Lysophosphatidic acid (LPA) has multiple functions, including smooth muscle contraction, cell proliferation and differentiation, angiogenesis and platelet aggregation through binding to its G protein-coupled receptors.[1–4] Recently, LPA has also been reported to play an important role in regulating immune responses with a control on migration and activation of lymphocytes and dendritic cells.[5] LPA is produced by many kinds of cells, such as platelets, fibroblasts and ovarian cancer cells.[6,7] LPA is also produced from lysophospatidylcholine extracellularly by plasma lysophospholipase D (lysoPLD),[7–9] which is known to be identical to the soluble form of tumor cell motility-stimulating factor autotaxin (ATX).[9] ATX can supply LPA directly and more effectively to its receptors compared to the intracellular phospholipid biosynthesis pathways, and plasma LPA concentration strongly correlates with activity and

concentration of ATX in serum.[10–12] In addition, depletion of ATX from plasma resulted in dramatical decrease in the production of LPA,[13] showing that ATX is responsible for bulk LPA production in blood. In human, ATX activity exists in serum and plasma, follicular fluid,[14] saliva and amniotic fluid, although its source is unknown. LPA in follicular fluid is reported to stimulate the oocyte maturation.[15] Recently, ATX was reported to be required for blood vessel formation in embryo by producing LPA.[13,16] ATX activity in human serum was higher in female than in male,[17] and increased during pregnancy paralleled with gestational weeks and decreased to near the non-pregnant level soon after delivery,[9,18] suggesting the higher plasma LPA concentration during pregnancy and possible roles of ATX in pregnancy by producing LPA. Interestingly, the ATX activity is higher in threatened pre-term delivery patients than in normal pregnant women.[9] This suggested the possible involvement of ATX in human parturition through the higher plasma concentration of its resultant substance, LPA.

Autotaxin activity increases during pregnancy and decreases soon after the delivery, but the source of increased ATX during pregnancy is still uncertain. We hypothesized that the source of increased ATX might be placenta and investigated the expression of ATX in human placenta using immunohistochemical staining and polymerase chain reaction (PCR).

Methods

All sample collections and experimental procedures were conducted under the approval of the Ethical Committee of Medical Faculty, University of Tokyo. Informed consent was obtained prior to each sample collection.

Placenta Tissue Collection

Human placenta tissues for immunohistochemical staining and RNA analysis were obtained from the cases of legal pregnancy termination in the first gestational trimester (7–9 weeks, 5 cases, and 6–11 weeks, 9 cases, respectively) and the second gestational trimester, (18 weeks, 4 cases, and 18 weeks, 5 cases, respectively), and of elective cesarean section in the third gestational trimester (37–40 weeks, 4 cases, and 37–40 weeks, 11 cases, respectively) before the onset of labor. All the women did not have any pregnant complications such as recurrent miscarriage, pregnant induced hypertension or intrauterine infection.

Monoclonal Antibody (mAb) Against Human ATX

Rat anti-ATX/lysoPLD monoclonal antibody (2A12) was generated by the immunization of rat with a polypeptide (amino acids 58–182 of human ATX) at the Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan. The specificity and immunoreactivity for tissue section of the mAb were evaluated previously.[19–21]

Immunohistochemistry

Paraffin-embedded sections of placenta tissue, 7 μm thick on slides, were deparaffinized in xylene, hydrated through a graded series of ethanol, and then immersed in 3% hydrogen peroxide in 100% methanol for 30 min to inhibit endogenous peroxidase activity. To activate the antigens, the sections were boiled in 10 mM citrate buffer pH 6.0 for 15 min.

Being washed with Tris-buffered saline (TBS) (50 mM Tris–HCl, 150 mM NaCl, pH 7.6), the slides were incubated in 0.1% avidin—50 mM Tris–HCl for 10 min at room temperature (RT). Being washed with TBS, the slides were then incubated in 0.01% biotin—50 mM Tris–HCl for 10 min at RT (Biotin-blocking System; DAKO, Carpinteria, CA, USA). Being rinsed in TBS, the slides were incubated with normal rabbit serum for 30 min and incubated overnight at 4°C in humid chambers with primary antibodies to ATX/lysoPLD (2A12, dilution 1:160). Being washed with TBS, the sections were incubated with biotinylated rabbit anti-rat immunoglobulin (1:1000) (DAKO) for 1 hr. Being washed with TBS again, the slides were treated with peroxidase-conjugated streptavidin (1:500) (DAKO) for 30 min and developed by immersion in 0.01% H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride. Light counterstaining with Mayer's hematoxylin was performed. All cases had a negative control that was run simultaneously with the test slide, in which control rat IgG (BD Biosciences Pharmingen, San Jose, CA, USA) was used as the primary antibody.

Reverse Transcription and Real-time Polymerase Chain Reaction

Total RNA of placenta tissues at the first, second, and third gestational trimesters was extracted using

RNAeasy Mini Kit (Qiagen, Hilden, Germany). The concentration of extracted RNA was measured spectrophotometrically. A reverse transcription (RT) was performed using the ReverTraAce kit (Toyobo, Osaka, Japan) according to the manufacture's instruction. A real-time PCR was carried out using the LightCycler thermal cycler system (Roche Diagnostics, Lewes, UK), using the following primer pairs: ATX (273 bp)- forward; 5'-GTTGCAAGGAAA CCTTTGGA-3',[22] reverse; 5'-CATGGTTGGCCTGAAG GTAT-3' [designed by entering ATX cDNA specific sequence on 'primer3' primer making software (http://workbench.sdsc.edu)], and beta-actin (113 bp)- forward; 5'-GAAATCGTGCGTGACATTAAGG-3',[23] reverse; 5'-TCAGGCAGCTCGTAGCTTCT-3'.[23] PCR was performed in a 20 μL volume containing a 5 ng sample of cDNA, 0.5 μM primers, 3 mM $MgCl_2$, and LightCycler FastStart Reaction Mix SYBR Green I DNA master mixture (Roche Diagnostics). Following 10 min of denaturing at 95°C, 40 cycles of amplification were conducted (for ATX: 95°C denaturation for 10 s, 57°C annealing for 10 s and 72°C extension for 11 s, and for beta-actin: 95°C denaturation for 10 s, 65°C annealing for 10 s and 72°C extension for 4 s). Beta-actin mRNA was quantified in each sample as an internal control to normalize the level of mRNA among samples. To confirm the amplification specificity, PCR products were assessed by melting curve analysis and 2% agarose gel electrophoresis. Direct sequencing revealed that the base-pair sequences of PCR products with ATX-primer pair and beta-actin-primer pair were identical to ATX and beta-actin, respectively.

Statistics

Autotaxin mRNA amounts in placenta tissues at the first, second, and third gestational trimesters were evaluated after compensation with beta-actin mRNA amounts. Statistical analysis was performed using Mann–Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

Results

The Local Distribution of ATX Protein in the First, Second, and Third Trimester Placenta

Immunohistochemical staining using the specific mAb for ATX protein (2A12) was performed to investigate the local distribution of ATX protein. The most positive staining was observed in trophoblasts including villous syncytiotrophoblasts, villous cytotrophoblasts and cytotrophoblast cell columns in all stage of pregnancy [first (n = 5, 7–9 weeks), second (n = 4, 18 weeks) and third (n = 4, 37–40 weeks) trimester], although staining patterns of villous trophoblast were not constant at the third trimester. The pictures shown are representative of the cases in each trimester (Fig. 1).

The mRNA Expression of ATX in the First, Second, and Third Trimester Placentas

To investigate the amounts of ATX mRNA in the first, second, and third placenta tissues, we conducted a real-time PCR for ATX. The amounts of ATX mRNA after compensation with beta-actin mRNA amounts in the second trimester placenta (n = 5, 18 weeks) and the third trimester placenta (n = 11, 37–40 weeks) were higher than those in the first trimester placenta (n = 9, 6–11 weeks) (Fig. 2).

Discussion

The activity of ATX, in human serum, increases in normal pregnant women paralleled with gestational weeks and decreases to near the non-pregnant level soon after delivery.[9] In this study, by employing the immunohistochemistry with a specific antibody, we demonstrated the expression of ATX in the placenta of all gestational trimesters. At all gestational trimesters, the most marked positive staining of ATX was identically observed in trophoblasts including villous syncytiotrophoblasts, villous cytotrophoblasts and cytotrophoblast cell columns. These findings suggested that ATX observed at higher activity in the serum during pregnancy was mostly originated from the placental trophoblasts. The staining patterns of villous cytotrophoblasts were not constant at the third trimester. This might be due to the decrease of bioactivities of villous cytotrophoblasts at term pregnancy.

In human placenta, villous cytotrophoblasts proliferate, aggregate, and form cytotrophoblast cell columns at the tip of anchoring villi. Cytotrophoblasts attaching to the uterine wall differentiate into extravillous cytotrophoblasts and invade into the decidual interstitium and further into spiral arteries. By replacing vascular endothelial cells, extravillous cytotrophoblasts play a central role in the formation of

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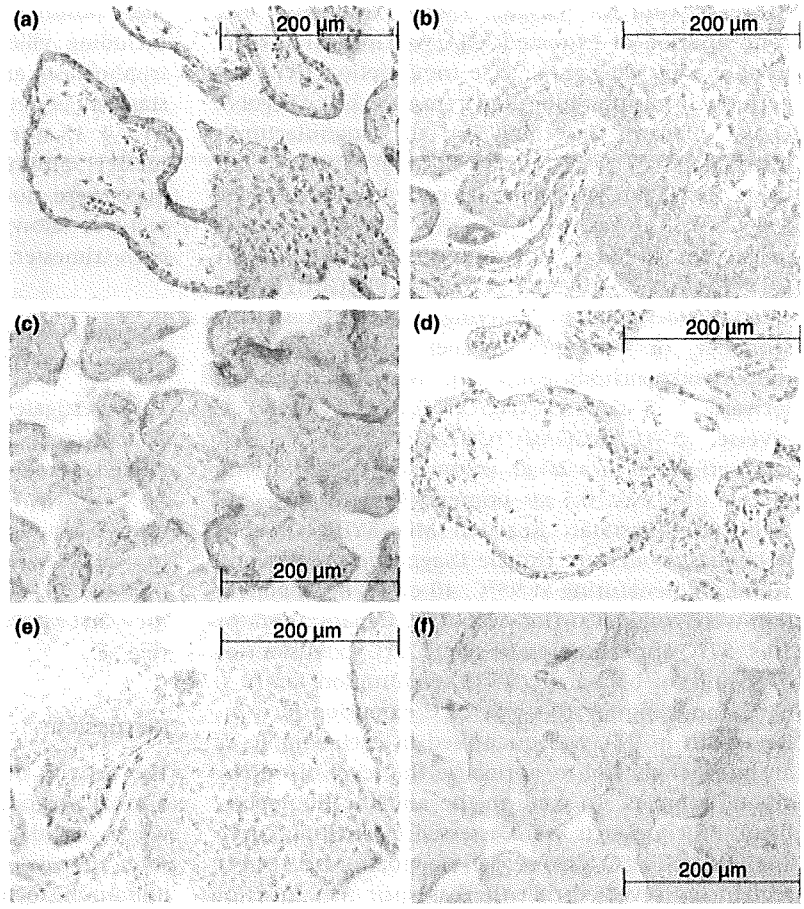

Fig. 1 The local distribution of autotaxin (ATX) protein in the first, second, and third trimester placenta. Immunohistochemical study was conducted to see the distributions of ATX protein in placentas at the first (a, d), second (b, e), and third (c, f) trimester using specific mAb to ATX (2A12) (a–c). No staining was detected in negative controls using isotype matched mouse IgG (d–f). Scale bar = 200 μm. The pictures shown are representative of the cases in each trimester.

the inter-villous cavity and the reconstruction of vascular structure of the uterine wall. In other way, some of villous cytotrophoblasts fuse, differentiate into villous syncytiotrophoblasts, and play multiple functions, such as substance exchange and hormone secretion in placenta.

Soluble ATX, which is produced by shedding of ATX, is the most important molecule for the regulation of extracellular concentration of LPA.[7–12] LPA has multiple functions including cellular proliferation, cellular differentiation, angiogenesis, and immune regulation.[1–5] ATX expressed by trophoblasts should be converted to soluble ATX, and then may work as a regulator for trophoblast proliferation, trophoblast differentiation, vascular remodeling, and feto-maternal immune interaction in placenta through the function of LPA.

Lysophosphatidic acid may also regulate implantation. Of the specific receptors to LPA, deletion of LPA_3 in mice was reported to result in reduced litter size.[19] Interestingly, LPA_3 expression in uterus was strictly up-regulated during early pregnancy and then returned to the basal level through the end of pregnancy.[24] LPA regulates immune responses with a control on migration and activation of lymphocytes and dendritic cells.[5] In implantation, early embryo is thought to need local inflammation to penetrate and invade in decidual tissue. ATX might work in implantation by producing LPA that may induce inflammation through the activation of lymphocytes and dendritic cells.

The activity of ATX was the highest at the third trimester of pregnancy and to a higher extent in patients in threatened pre-term delivery.[9] As for the receptors for LPA, LPA_1, mRNA level was identified in cultured myometrial cells from pregnant women; and in rat uterine LPA_1, mRNA level was elevated at the end of gestation.[25] Intravenous injection of LPA

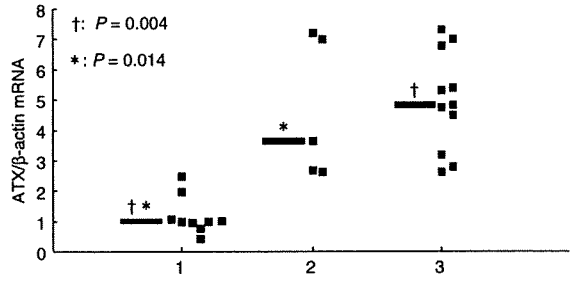

Fig. 2 The mRNA expression of ATX in the first, second, and third trimester placentas. Real-time PCR was conducted to investigate the amounts of ATX mRNA in the first, second, and third placenta tissues. The amounts of ATX mRNA after compensation with beta-actin mRNA amounts in the second trimester placenta (Lane 2; $n = 5$, 18 weeks, median 3.7, 5-percentile 2.6, 95-percentile 7.2) and the third trimester placenta (Lane 3; $n = 11$, 37–40 weeks, median 4.8, 5-percentile 2.7, 95-percentile 7.2) were higher than those in the first trimester placenta (Lane 1; $n = 9$, 6–11 weeks, median 1.0, 5-percentile 0.6, 95-percentile 2.3) ($P = 0.014$ (second versus first); $P = 0.004$ (third versus first). Data are represented by dots and median values are expressed by horizontal bars.

induced an immediate rise in intrauterine pressure, like prostaglandin F2 alpha.[26] Oxytocin receptor gene expression in the myometrial cells in primary culture from women in late gestation was up-regulated by the addition of LPA.[27] These findings suggested the roles of ATX and its product, LPA, in induction of labor.

Autotaxin activity in plasma increases paralleled with gestational weeks. To clarify the mechanism how ATX activity in plasma increases paralleled with gestational weeks, we performed real-time PCR to quantify mRNA of ATX in placenta at the first, second, and third trimester. As ATX protein exists in blood, we did not quantify ATX protein by Western blotting in placenta tissues, which contain much amount of blood. The real-time PCR revealed that the mRNA amounts of ATX in placenta tissues paralleled with gestational weeks, i.e. the ATX level in plasma, suggesting that the increase in the ATX level during pregnancy[9,18] is due to the increase in the production of mRNA by each cell as well as due to the increase in size of placenta, mostly in the number of trophoblasts, which might cause the increase in total amount of ATX protein produced by placenta.

In summary, we identified the molecules of ATX in placenta tissue, especially in trophoblasts and found that mRNA production of ATX in placenta tissues paralleled with gestational weeks, i.e. the ATX level in plasma. These findings suggest that trophoblasts might produce ATX and its bioactive resultant substance, LPA, paralleled with gestational weeks. This is the first report that proves controlled expression of ATX in human placenta.

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British Journal of Cancer (2009) 101, 145–148

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The oncogenic mutation in the pleckstrin homology domain of AKT1 in endometrial carcinomas

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BACKGROUND: The phosphatidylinositol 3′-kinase (PI3K)–AKT pathway is activated in many human cancers and plays a key role in cell proliferation and survival. A mutation (E17K) in the pleckstrin homology domain of the *AKT1* results in constitutive AKT1 activation by means of localisation to the plasma membrane. The *AKT1* (E17K) mutation has been reported in some tumour types (breast, colorectal, ovarian and lung cancers), and it is of interest which tumour types other than those possess the *E17K* mutation.

METHODS: We analysed the presence of the *AKT1* (E17K) mutation in 89 endometrial cancer tissue specimens and in 12 endometrial cancer cell lines by PCR and direct sequencing.

RESULTS: We detected two *AKT1* (E17K) mutations in the tissue samples (2 out of 89) and no mutations in the cell lines. These two *AKT1* mutant tumours do not possess any mutations in *PIK3CA*, *PTEN* and *K-Ras*.

INTERPRETATION: Our results and earlier reports suggest that *AKT1* mutations might be mutually exclusive with other PI3K–AKT-activating alterations, although *PIK3CA* mutations frequently coexist with other alterations (such as *HER2*, *K-Ras* and *PTEN*) in several types of tumours.

British Journal of Cancer (2009) **101,** 145–148. doi:10.1038/sj.bjc.6605109 www.bjcancer.com
Published online 2 June 2009

Keywords: *AKT1*; mutation; PI3-kinase; endometrial carcinoma

The AKT serine/threonine kinases regulate diverse cellular processes, including cell survival, proliferation, invasion and metabolism (Vivanco and Sawyers, 2002). The phosphatidylinositol 3′-kinases (PI3Ks) are widely expressed lipid kinases that catalyse the production of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates AKT by recruitment to the plasma membrane through direct contact of its pleckstrin homology (PH) domain (Stokoe *et al*, 1997; Lemmon and Ferguson, 2000). Constitutive PI3K–AKT pathway activation can result from various types of alterations in this pathway, including mutation or amplification of receptor tyrosine kinases (such as *EGFR* and *HER2*), mutation of *Ras*, mutation or amplification of *PIK3CA* (the p110α catalytic subunit of PI3K) and inactivation of the tumour suppressor gene, *PTEN* (Yuan and Cantley, 2008). In addition to amplifications in multiple AKT isoforms in pancreatic, ovarian and head and neck cancers (Engelman *et al*, 2006), a somatic missense mutation in the PH domain of *AKT1* (E17K) was identified in breast, colorectal, ovarian and lung cancers and in melanoma (Carpten *et al*, 2007; Bleeker *et al*, 2008; Davies *et al*, 2008; Malanga *et al*, 2008). However, the *AKT1* mutation has not been identified in hepatocellular, gastric and pancreatic cancers, leukemia, as well as in glioblastoma multiforme (Bleeker *et al*, 2008; Cao *et al*, 2008; Kim *et al*, 2008; Mahmoud *et al*, 2008; Mohamedali *et al*, 2008; Riener *et al*, 2008; Zenz *et al*, 2008). Further study is required to fully understand which tumour types take advantage of *Akt1* (E17K) mutations to activate the PI3K–AKT pathway.

We reported earlier that *PIK3CA* mutations frequently coexist with other PI3K-activating alterations in breast (with *HER2* and *HER3*) and endometrial cancers (with *PTEN* and *K-Ras*), and that mutant p110α combined with mutant Ras efficiently transformed immortalised human mammary epithelial cells (Oda *et al*, 2005, 2008). Frequent overlapping mutations of *K-Ras* and *PIK3CA* were also reported in colorectal cancer (Parsons *et al*, 2005). Although coexistent mutations of *AKT1* and *PIK3CA* mutations are suggested to be infrequent in breast cancer (Carpten *et al*, 2007; Bleeker *et al*, 2008), it remains to be elucidated whether *AKT1* mutations are mutually exclusive with all the other PI3K–AKT-activating alterations in various tumour types.

Endometrial cancer is one of the tumour types in which the PI3K–AKT pathway is frequently activated by alterations of various genes. The frequency of mutations for *PTEN*, *PIK3CA* and *K-Ras* in endometrial cancer is reported as 54, 28 and 11%, respectively (Yuan and Cantley, 2008). In this study, we screened 89 endometrial carcinoma specimens and 12 endometrial carcinoma cell lines for mutations in *Akt1* (E17K) and analysed whether *AKT1* mutations coexist with any mutations in *PTEN*, *PIK3CA* and *K-Ras*.

MATERIALS AND METHODS

Tumour samples and genomic DNA

Surgical samples were obtained from 89 patients with primary endometrial carcinomas who underwent resection of their tumours

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Received 16 February 2009; revised 21 April 2009; accepted 27 April 2009; published online 2 June 2009

at the University of Tokyo Hospital. All patients provided informed consent for the research use of their samples and the collection, and the use of tissues for this study was approved by the appropriate institutional ethics committees. Genomic DNA was extracted by a standard SDS-proteinase K procedure. Patient characteristics (histology, tumour grade and stage) are available in Supplementary Table 1. A detailed distribution of the histological subtypes was as follows; 81 (90%) endometrioid adenocarcinomas, 3 adenosquamous carcinomas, 1 clear cell carcinoma, 1 squamous cell carcinoma and 3 mixed carcinomas.

PCR and sequencing

The primer sequences and PCR conditions of exon 4 of the *AKT1* gene are forward: 5′-CACACCCAGTTCCTGCCT G-3′ and reverse: 5′-CCTGGTGGGCAAAGAGGGCT-3′. The PCR amplifications were with denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s and final extension at 72°C for 10 min. The PCR conditions and the PCR primers for *PIK3CA* (exons 9 and 20), *PTEN* (exons 1–9) and *K-Ras* (exons 1 and 2) were described earlier (Minaguchi *et al*, 2001; Samuels *et al*, 2004; Oda *et al*, 2008). The PCR products were sequenced using the BigDye (Applied Biosystems, Foster City, CA, USA) terminator method on an autosequencer.

Cell lines

In this study, AN3CA, KLE, HEC-1B and RL95-2 were obtained from the American Type Culture Collection (Manassas, VA, USA) and HHUA was obtained from the RIKEN CELL BANK (Tsukuba, Japan). Ishikawa3-H-12 was a generous gift from Dr Masato Nishida (Kasumigaura Medical Center, Ibaraki, Japan). HEC-6, HEC-50B, HEC-59, HEC-88, HEC-108 and HEC-116 cell lines were also analysed in this study. The culture condition of all these cell lines was described earlier (Oda *et al*, 2008).

DNA methylation analysis

Bisulphite treatment was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). As described earlier (Ehrich *et al*, 2006), we used Sequenom's MassARRAY platform to perform quantitative methylation analysis of multiple CpG sites for *PTEN* in 53 endometrial tumour specimens (Sequenom, San Diego, CA, USA). Chromosomal localisation of CpG islands for *PTEN* and the primer sequences in this study are shown in Supplementary Figure 1.

Immunohistochemistry (IHC)

Immunohistochemistry for PTEN on 4-μm tissue sections was performed and evaluated as described earlier (Minaguchi *et al*, 2007). In this study, the anti-PTEN Rabbit monoclonal antibody (138G6) (Cell Signaling, Beverly, MA, USA) was applied at a dilution of 1 : 100.

Single nucleotide polymorphism (SNP) array

Single nucleotide polymorphism array was performed in the two *AKT1* mutant tumours with tumour DNA. Experimental procedures for GeneChip were performed according to GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA), using a Human mapping 50K Array Xba I (Affymetrix).

RESULTS AND DISCUSSIONS

The sequencing analysis for exon 4 of the *AKT1* gene in 89 tumour tissue samples of endometrial carcinomas showed the point mutation of G to A at nucleotide 49 (E17K) in two tissue samples (2.2%) (Figure 1). Both of the tumours were well-differentiated endometrioid adenocarcinomas with positive oestrogen receptor and progesterone receptor, suggesting that these two tumours are oestrogen dependent (corresponded to type I endometrial cancer). No mutations were detected in the 12 endometrial cancer cell lines.

Thereafter, we attempted to figure out the exclusivity of *AKT1* mutations and other PI3K–AKT-activating mutations (Supplementary Table 1). The genotypic pattern of the four genes (*PTEN*, *PIK3CA*, *K-Ras* and *AKT1*) in 97 endometrial carcinomas (85 tumour tissue samples and 12 cell lines) was shown in Table 1. Coexistence with other mutations is frequently observed in the *PIK3CA* mutant (28 of 34; 82%) and in the *K-Ras* mutant (13 out of 17; 76%) tumours, but the two *AKT1* mutant tumours do not possess any mutations in *PTEN*, *PIK3CA* and *K-Ras*. As PI3K and

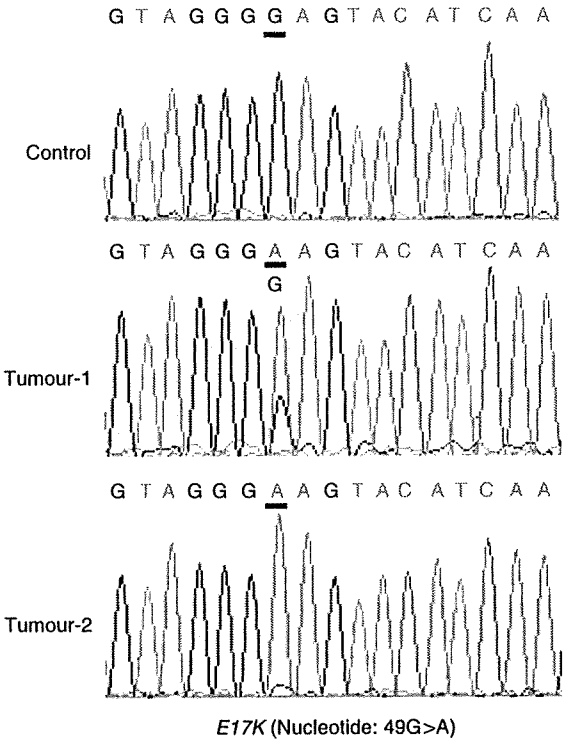

Figure 1 The sequence traces of two tumours and a normal control for exon 4 of *AKT1*. The *E17K* mutation is caused by a missense mutation (G to A) indicated. In tumour-2, the level of the mutant band (A) is much higher than that of the wild-type band (G). It is possible that this weak band is derived from DNA of normal cells and that the tumour might lose one allele at this locus.

Table 1 PI3K–AKT activating mutations and their coexistence in 97 endometrial cancers

	n (%)
Wild-type	24 (25)
AKT1 mutation alone	2 (2)
K-Ras mutation alone	4 (4)
PIK3CA mutation alone	6 (6)
PTEN mutation alone	30 (31)
Double mutations of *K-Ras* and *PIK3CA* (w/o *PTEN* mutation)	2 (2)
Double mutations of *K-Ras* and *PTEN* (w/o *PIK3CA* mutation)	3 (3)
Double mutations of *PIK3CA* and *PTEN* (w/o *K-Ras* mutation)	18 (19)
Triple mutations of *K-Ras*, *PIK3CA* and *PTEN*	8 (8)

PI3K, phosphatidylinositol 3′-kinase. Wild-type, no mutations in *PTEN*, *PIK3CA*, *K-Ras* and *AKT1*.

PTEN are competitive for PIP3 production, the *PIK3CA* mutation might require another upstream input or PTEN loss itself to fully activate the PI3K–AKT pathway. As AKT1 (E17K) functions downstream of PTEN and shows constitutive localisation to the plasma membrane in the absence of serum stimulation (Carpten *et al*, 2007), mutant *AKT1* (E17K) alone might be sufficient for complete activation of this pathway.

We also analysed DNA methylation and protein expression of PTEN, as hypermethylation and loss of heterozygosity (LOH) are other mechanisms to inactivate PTEN (Teng *et al*, 1997; Blanco-Aparicio *et al*, 2007). Quantitative analysis of DNA methylation using Sequenom's MassARRAY platform did not find promoter hypermethylation of *PTEN* in all the 53 samples that were examined (Supplementary Figure 2 and Supplementary Table 2), including the two *AKT1* mutant tumours. Although *PTEN* methylation had been reported in 18% of endometrial carcinomas (Salvesen *et al*, 2001), Zysman *et al* (2002) suggested that the pseudogene on chromosome 9 (Genbank accession number: AF040103), not *PTEN*, is predominantly methylated in endometrial carcinomas. In IHC, both tumours with the *AKT1* mutation were stained positively for PTEN in the cytoplasm, whereas all the four tumours with multiple frameshift mutations in *PTEN* were stained negatively (Supplementary Figure 3). We evaluated the chromosomal imbalances in the two *AKT1* mutant tumours, using SNP array (with more than 50 000 SNPs). Single nucleotide polymorphism array analysis showed that the two *AKT1* mutant tumours do not show copy number changes in the locus of *PTEN* (10q23.1) (data not shown). These data also support the fact that *AKT1* mutations are mutually exclusive with PTEN inactivation.

We found multiple *PTEN* mutations in 13 out of 85 clinical specimens and in 8 out of 12 endometrial cell lines (Supplementary Table 1), whereas LOH of *PTEN* was reported approximately at 30% in endometrial carcinomas (Toda *et al*, 2001). Thus, biallelic *PTEN* inactivation might be achieved through either biallelic mutations or monoallelic mutation with LOH in endometrial carcinomas. Considering the correlation between *PTEN* mutations and microsatellite instability (MSI) in endometrial carcinomas (Bilbao *et al*, 2006), it would be of interest to analyse whether *AKT1* and the other mutations in the PI3K pathway genes are also associated with MSI.

To date, *AKT1* (E17K) mutations have been reported in breast (25 out of 427; 5.9%), colorectal (4 out of 243; 1.6%), lung (4 out of 636; 0.6%) and ovarian cancers (1 out of 130; 0.8%) and in melanoma (1 out of 202; 0.5%). Breast, colorectal and endometrial cancers are the tumour types that frequently possess *PIK3CA* mutations (Campbell *et al*, 2004; Samuels *et al*, 2004; Oda *et al*, 2005). In lung cancer, the *AKT1* mutation was detected only in squamous cell carcinomas and not in any adenocarcinomas, which is in agreement with the higher incidence of *PIK3CA* mutations or amplifications in squamous cell carcinomas than adenocarcinomas (Kawano *et al*, 2006, 2007; Malanga *et al*, 2008). These data suggest that the *AKT1* mutation might occur in a tissue-specific manner and is more associated with the tumour types with frequent *PIK3CA* alterations.

ACKNOWLEDGEMENTS

We thank Kaoru Nakano, Hiroko Meguro, Shingo Yamamoto, Akira Tsuchiya and GLab Pathology Center Co., Ltd. for their technical support. We thank Dr Masato Nishida for Ishikawa3-H-12 cell line. This work was supported by KAKENHI, the Grant-in-aid for Scientific Research (C) in Japan, number 19599005 (to K Oda).

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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Effect of Progesterone on HLA-E Gene Expression in JEG-3 Choriocarcinoma Cell Line

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Keywords
human leukocyte antigen-E, JEG-3, progesterone, trophoblast

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Submitted November 22, 2008; accepted December 29, 2008.

Citation
Huang Z, Hyodo H, Fujii T, Nagamatsu T, Matsumoto J, Kawana K, Yamashita T, Yasugi T, Kozuma S, Taketani Y. Effect of progesterone on HLA-E gene expression in JEG-3 choriocarcinoma cell line. Am J Reprod Immunol 2009; 61: 221–226

doi:10.1111/j.1600-0897.2008.00684.x

Problem
Among class Ib human leukocyte antigen (HLA) molecules, HLA-E is known to be a major ligand of CD94/NKG2 receptor on natural killer (NK) cells, and to play a pivotal role in recognition of extravillous trophoblasts (EVTs) by maternal immune cells. However, it is scarcely known how HLA-E expression is regulated in EVTs.

Method of study
In this study, we investigated whether progesterone, an essential hormone in maintaining pregnancy, regulated HLA-E expression in EVT-like cell line, JEG-3. HLA-E mRNA amount in cultured JEG-3 cells was assessed by real-time PCR and cell-surface HLA-E protein was analyzed by flowcytometry.

Results
Real-time PCR showed 3.5-fold increase 1 hour after the addition of 1000 ng/ml progesterone. This response was dimished by the addition of RU486, an antagonist for progesterone receptor. Flowcytometry indicated that 1000 ng/ml progesterone slightly enhanced HLA-E expression on the surface of JEG-3.

Conclusion
These results suggest that progesterone up-regulates HLA-E expression in JEG-3 cells through the pathway mediated by progesterone receptor. Our findings might give a new insight into immunomodulatory function of progesterone at fetomaternal interface.

Introduction

In human placenta, expression of human leukocyte antigen (HLA) molecules on trophoblasts is highly restricted. The interaction between HLA molecules on trophoblasts and the corresponding receptors on decidual lymphocytes is considered to be an immunological key for successful pregnancy. Extravillous trophoblasts (EVTs) come into direct contact with maternal immune cells in their migrating process into the decidua. EVTs do not express HLA class II and class Ia molecules except for HLA-C.[1] Instead, they express low polymorphic class Ib molecules such as HLA-E, -F and -G.[2,3] Among class Ib molecules on trophoblasts, HLA-G has been most extensively studied about its molecular distribution in placenta as well as its function based on the hypothesis that HLA-G has specialized function in the immunological tolerance to fetus because of the unique localization restricted to fetomaternal interface in placenta. On