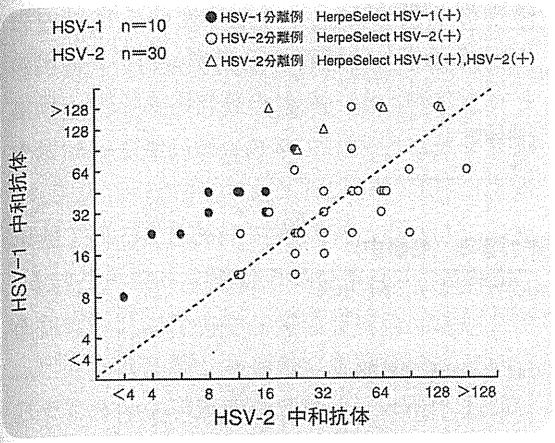

図8 再発型 経腟分娩例 妊娠末期中和抗体価

ているが，今後の検討が必要である。

3 分娩様式の選択（表11）

分娩時に外陰病変があれば帝王切開を行う。外陰病変がなくても初感染では発症より1カ月以内，再発では発症より1週以内はやはり帝王切開を行う。初感染では1カ月以上，再発では1週以上経ていて外陰病変がなければ経腟分娩を行う。初感染の場合，発症から1カ月以上経つとIgGによる中和抗体が産生されるようになると考えられることと，初感染性器ヘルペス合併妊婦でHSVの分離期間は2週間以内が多いが，稀に1カ月間続いた例があったのでこの管理法を考案した。なお，新生児は出生時に目，鼻，口，耳，性器よりHSVの分離を行うとともに臍帯血のIgG抗体，IgM抗体を測定する。新生児は7日以上は入院管理として新生児ヘルペスの早期発見に努める。このような管理方法を行ってきて性器ヘルペス合併妊婦から新生児ヘルペス児は1例も発症していない。ただし，この管理方式はアシクロビルなどの有効な抗ウイルス薬が開発される前に作成したものである。抗ウイルス薬を使用することにより治癒までの期間が著しく短縮されているので，初発の場合でも病変が治癒しウイルス分離が陰性ならば必ずしも1カ月にこだわる必要はなくてもよいであろう。また，再発例についても中和抗体が十分あって抗ウイルス薬を投与して治癒を促進し，ウイルス分離が陰性ならばこの管理方式によることもないであろう。いずれにせよ，抗ウイルス薬使用による管理方式は今後の課題である。

表11 性器ヘルペスの合併妊娠の管理：分娩様式の選択

①分娩時に外陰病変あり・・・・・・・・・・	：帝切
②分娩時に外陰病変なし	
a．初感染・・・・・・発症より1カ月以内	：帝切
発症より1カ月以上	：経腟
b．再発型または非初感染初発	
・・・・・・発症より1週間以内	：帝切
1週以上	：経腟

XIV. “再発”性器ヘルペス合併妊婦の管理

妊娠中に性器ヘルペスが再発した妊婦や妊娠する前から再発性器ヘルペスを有している妊婦は，どのように管理するかについてはいろいろな考え方が錯綜している。その理由は，分娩時再発例の経腟分娩による新生児ヘルペスの発症が0～2%と報告により多少差はあるが，いずれにしてもかなり低率であるので重要視しない考えがある一方で，新生児ヘルペスの悪い予後を考えると2%を重大ととらえる考えがあるからであろう。

オランダでは，分娩時に再発していても経腟分娩を行っているようだし，前述のProberの報告[32]でも再発例では新生児ヘルペスを発症することはまずないと報告しているので，分娩時に再発していても経腟分娩による新生児ヘルペス発症のリスクはかなり低いとみてよいであろう。

英国の2007年のガイドラインをみると，分娩時に再発している場合，産婦に対して新生児ヘルペスの発症のリスクは非常に低いことを告げるようにするべきで，帝王切開を

ルーチンに進めることはないという立場をとっている[34]。このような考えに至る背景には，英国では新生児ヘルペスは6万出生に対して1例と非常に低い頻度であることも関連しているであろう。

しかし，一方では新生児ヘルペスを恐れるあまり病変の有無と関係なくすべて帝王切開分娩にしてしまうこともあるようであるが，筆者らは米国のように[35]病変があれば帝王切開を選択することにしている。病変の有無により分娩様式を選択するという考えに従えば分娩時の再発を抑えれば経腟分娩が可能になる。米国では，性器ヘルペスによる帝王切開がかなり頻繁に行われているようであり，これに対して性器ヘルペスの再発抑制療法を妊娠36週より行って再発を抑制して帝王切開しないで済むようにしようという管理方式が提案された。再発抑制療法とは，妊娠36週からアシクロビル200 mg×4回，またはバラシクロビル500 mg×2回を毎日服用する方法である。1日量としては，非妊婦に行われている再発抑制療法の2倍量が用いられている。最近，Sheffieldらは5つの報告のメタアナリシスを行ってその効果について検証している。それによると，確かに分娩時の再発や再発の前兆は減少し，したがって帝王切開例も有意に減少している[36]。最近，米国のACOGもこの方法を推奨している[37]。今のところはアシクロビルによる新生児の副作用といわれている好中球減少もないという。

しかし，一方で分娩時に再発していて経腟分娩を行っても新生児ヘルペスは発症しないというオランダの経験や，前述のProberの報告[32]，そして筆者らも再発性器ヘルペス例についてほとんどの例で経腟分娩を行ったが新生児ヘルペスの発症例はなかったなどの経験もあり，妊娠36週からの抑制療法を全例について行うことには賛成できない。

また，バラシクロビルを用いて妊娠36週より抑制療法を行っても分娩前7日以内のHSV排泄率と分娩時の発症率は，プラセボ群と有意差がなかったとの報告もある[38]。母体に投与されたアシクロビルは高濃度に胎児や羊水に移行することが知られているので，1カ月にもわたる抑制療法が長期的にみて胎児へ腎機能(アシクロビルは腎より排泄される)や骨髄機能に影響がないのかやはり不安であるので，今後厳重な追跡は必要であろう[39]。

最近，Hollierらが7つの報告のメタアナリシスを行っている。再発抑制療法を行っても新生児ヘルペスの発生を減少させたという証拠はなく，また新生児の長期の副作用に関する検討も十分ではないとしている[40]。

ただ，抑制療法は妊娠末期に子宮頸管からのHSV分離が陽性の例や妊娠10カ月に入ってから頻繁に再発を繰り返す例，そして再発を恐れて精神的に不安定になっている妊婦などには考慮してもよいであろう。

XV. 分娩管理の新しい試み

再発性器ヘルペスを有する妊婦に対する前述の抑制療法は，いつ再発するかわからないので妊娠36週から分娩まで行っているのである。一方，臨床症状から再発を疑って帝王切開したものの後から振り返ると約2/3は誤診していたということも前述のとおりである。これらは，分娩時(入院時)に性器にHSVが感染していることを正確に，そして迅速に知ることのできる方法がないことに起因している。

筆者らは，迅速で感度・特異度ともによい核酸増幅法であるLAMP法が性器ヘルペスの診断に有効であることを確認した。この方法は2時間以内に結果を出せ，培養法とほぼ同じ感度である。この迅速検出法を用いて性器ヘルペス合併妊婦に対して新しい管理方法を案出した。まず入院時に外陰と子宮頸管か

らLAMP法にてHSV-DNAの検出を行い陰性の場合は経腟分娩を行う。陽性の場合は母体の中和抗体が8倍未満か，8倍以上でもウイルス量が多い場合は帝王切開で分娩させるが，ウイルス量が少ない場合で中和抗体が16倍以上あれば経腟分娩を試みる。この16倍以上という抗体価の評価については症例を重ねて検討する必要がある。出生した児については，ただちに目，鼻，口，耳，性器についてLAMP法を施行し，陽性ならばただちに抗ウイルス療法を行う。陰性ならば経過観察とする。産褥3日目にも母体の外陰と子宮頸管と児について検査を行う[41]。

この管理方法はまだ始まったばかりであるが，現在のところ新生児ヘルペス発症例はない。LAMP法が臨床の場で簡単に行うことができれば，再発性器ヘルペスのある妊婦について従来の経験的な管理法や36週からの盲目的な抑制療法を行わなくても母子感染の予防は可能となろう。

●おわりに

現在わが国では，性器ヘルペスを診断するための感度・特異度ともに優れた検査法がないために正確な診断が難しい。さらに，型特異抗体の測定もできないので，性器ヘルペスの感染病態も正しく診断できない。したがって，性器ヘルペス合併妊娠における精度の高い管理法はできないのが現状である。これらの方法が保険で使用できるようになることを切望している。

そして，最後に最も重要なことは，新生児ヘルペスを出生した母体の約70%は無症候であったということである。いかにこのようなリスクのある例を抽出して，HSVの母子感染を予防するかが今後の大きな課題である。一つの方法として，妊婦とその夫についてHSVの（型特異）抗体を検出しておいて，もし妊婦が抗体陰性で夫が陽性の場合，妊娠中期以降の性行為を禁止するかコンドームを使用させてHSVの感染を予防するなどの試みが提案されている[42]。ただ，全妊婦にスクリーニングを行うこの試みは費用対効果を考えると新生児ヘルペスの少ない地域では必ずしも適切な方策とはいえない[43]。いずれにせよ，新生児ヘルペス発症のハイリスク妊婦が特定できるような方策の確立が望まれる。

（川名　尚・大貫 裕子・西井　修）

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Review Article

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Human papillomavirus vaccines: current issues & future

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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

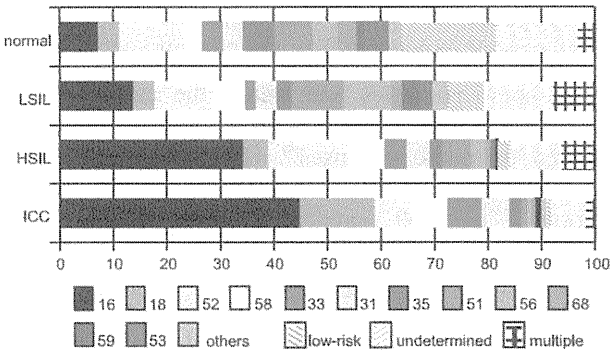

Fig. 1. HPV type distribution in cervical neoplasia in Japan[18]. HPV16 and 18 are the most common types in invasive cervical cancer (ICC) while more than 40 per cent of the invasive cancer is associated with the other types in Japan. HPV52 is the most common type in female with normal cytology in Japan[18].

(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

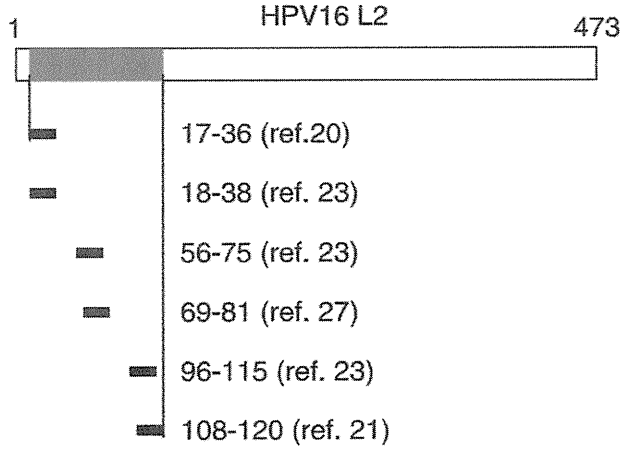

Fig. 2. Broad-spectrum neutralization epitopes of HPV16 L2 capsid protein. Many studies reveals linear epitope cross-neutralizing infection with many HPV types in L2 capsid protein. Each epitope includes amino acid conserved regions between genital HPV types and has potential of neutralization of HPV 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

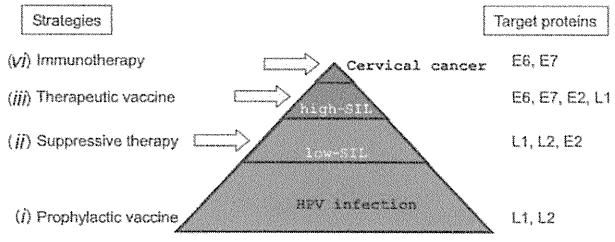

Fig. 3. Possible strategies utilizing immunological responses to HPV proteins for HPV-associated lesion and the target viral proteins for each strategy. (*i*) vaccines for the prevention of infection in uninfected females, (*ii*) vaccines for the reduction of viral load at the cervical mucosa in females 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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Identification of nucleolin as a protein that binds to human papillomavirus type 16 DNA

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ABSTRACT

Transcription, replication, and segregation of human papillomaviruses (HPVs) are regulated by various host factors, but our understanding of host proteins that bind to the HPV genome is limited. Here we report the results of a search of cellular proteins that can associate with specific genomic regions of HPV type 16 (HPV16). We found that human nucleolin, an abundant nucleolar protein, was preferentially captured *in vitro* by an HPV16 genomic fragment from nucleotide positions (nt) 531–780. Electrophoretic mobility shift assays with a bacterially expressed nucleolin revealed that nucleolin bound to an HPV16 genomic region between nt 604 and 614 in a sequence-dependent manner. Chromatin immunoprecipitation analysis showed that both exogenous and endogenous nucleolin bound to a plasmid containing the HPV16 genomic region in HeLa cells. Furthermore, nucleolin associated with the HPV16 genome stably maintained in HPV16-infected W12 cells, suggesting that the nucleolin binding may be involved in the dynamics of the HPV genome in cells.

Introduction

Human papillomaviruses (HPVs), which are recognized as the causative agents of cervical cancer, have circular double-stranded DNA genomes with sizes close to 8 kbp [1]. HPV infects basal cells in the epidermis and its genome is maintained as episomes, whereas the viral genome amplification occurs in upper differentiating epithelium [2]. Due to the limited coding capacity of its small genome, HPV relies heavily on the function of host cell proteins for viral transcription, replication and segregation [3,4]. The transcription of the HPV genome is driven by two major promoters: in HPV type 16 (HPV16) genome, the early promoter P_{97} directs early gene transcription, while the late promoter P_{670} induces capsid expression. After initial unwinding of the replication origin by the coordinated action of the HPV E1 and E2 proteins, the HPV DNA replication progresses with the use of cellular replication proteins. For viral genome maintenance, the HPV genome is passively segregated by being tethered to host chromosomes, then passed into nuclei of daughter cells. Because of a lack of cell culture systems for efficient HPV propagation, molecular mechanisms of these processes are not fully understood.

Nucleolin is an abundant, ubiquitously expressed protein that is found in the nucleolus, the nucleoplasm, and on the cell surface, and is involved in regulation of ribosomal DNA (rDNA) transcription and the maturation of pre-ribosomal RNA [5]. In addition, nucleolin exerts several nuclear functions related to the transcription of several genes by the RNA polymerase II [6–8], genotoxic stress response [9], and chromosome congression in mitosis [10].

To gain new insights into how the dynamics of the HPV genome are regulated by host factors, we searched for cellular proteins that can bind to the two promoter regions of HPV16 using an unbiased proteomic approach. We describe the identification and characterization of nucleolin as an HPV16 genome-binding protein that may play a role in regulation of the HPV life cycle.

Materials and methods

Isolation of HPV16 genome-binding protein and its identification by peptide mass fingerprinting. Three HPV16 DNA fragments, I (nt 7791–120, 234 bp), II (nt 131–360, 230 bp), and III (nt 531–780, 250 bp), were generated by PCR using following primers: I forward, 5′biotin—TAC ATG AAC TGT GTA AAG GTT AGT CA-3′; I reverse, 5′-TGT GGG TCC TGA AAC ATT GCA GTT CTC TTT-3′; II forward, 5′biotin—AGA AAG TTA CCA CAG TTA TGC ACA GA-3′; II reverse, 5′-GTT CCA TAC AAA CTA TAA CAA TAA TGT CTA-3′; III forward, 5′biotin—CAA GAA CAC GTA GAG AAA CCC AGC TG-3′; III reverse, 5′-GTG TGT

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GCT TTG TAC GCA CAA CCG-3′. The biotinylated PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega) and coupled to Dynabeads M-280 streptavidin (Dynal, Norway) in a buffer consisting of 10 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, and 1 M NaCl. The HeLa nuclear extract was prepared by Dignam's procedure and incubated with the HPV16 DNA-coupled magnetic beads at 4 °C overnight. The beads were then washed three times in a wash buffer (10 mM Hepes, pH 7.9, 200 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, and 10% glycerol). The bound proteins were released from the beads in SDS-sample buffer by boiling for 5 min and fractionated in a 4–20% gradient SDS–polyacrylamide gel (Daiichi Pure Chemicals, Japan), followed by silver staining. The 95-kDa protein band bound to fragment III was excised from the gel and subjected to in-gel trypsin digestion. The resultant peptide mixtures were analyzed by MALDI-QIT-TOF MS (AXIMA-QIT, Shimazu Biotech, Japan). Mascot software (Matrix Science) was used for protein identification.

Electrophoretic mobility shift assay (EMSA). The full-length cDNA of human nucleolin was amplified by RT-PCR from mRNA of HeLa cells. The resultant cDNA sequence completely matched the nucleolin sequence in the GenBank (NM 005381). To generate an expression plasmid for glutathione *S*-transferase (GST) fused nucleolin, the cDNA fragment encoding nucleolin amino acid (aa) from 289 to 710 was amplified by PCR with the full-length nucleolin cDNA as a template, and cloned into pGEX-2TK (GE Healthcare). GST–nucleolin and GST were expressed in *Escherichia coli* and purified using a GSTrap HT column and an AKTAprime (GE Healthcare). The EMSA was performed as described previously [11]. The DNA/protein complex was separated on a 5% polyacrylamide gel and visualized by autoradiography on X-ray films.

Chromatin immunoprecipitation (ChIP) assay. An expression plasmid for N-terminally FLAG-tagged nucleolin (FLAG-nucleolin) was constructed by cloning the full-length cDNA of nucleolin into p3xFLAG-CMV10 (Sigma). The ChIP assay was performed as described previously [11] with some modifications. Briefly, HeLa cells were transfected with pGL3-P_{670} or pGL3-Basic (Promega) together with the FLAG-nucleolin expression plasmid or p3xFLAG-CMV10 using FuGENE6 (Roche). At 48 h after the transfection, the cells were fixed with 1% formaldehyde at 37 °C for 5 min, lysed and sonicated using a Bioruptor (Cosmobio, Japan). The sonicated extract was immunoprecipitated with specific antibodies that had been coupled to Dynabeads M-280 sheep anti-mouse IgG (Dynal). Antibodies used were anti-FLAG M2 (Sigma), anti-nucleolin (MS-3, Santa Cruz, or 4E2, Abcam), or control mouse IgG (Santa Cruz). After washing the beads, the immunoprecipitated DNA/protein complexes were eluted, and reverse cross-linked. DNA was purified and subjected to PCR for HPV16 DNA (from nt 501 to 670), 18S rDNA, or pGL3-Basic. PCR primers were as follows: HPV16 forward, 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′; HPV16 reverse, 5′-CAT CCT CCT CCT CTG AGC TGT CAT TTA ATT-3′; 18S rDNA forward, 5′-GCC TGG ATA CCG CAG CTA GGA ATA ATG G-3′; 18S rDNA reverse, 5′-TTG ATT AAT GAA AAC ATT CTT GGC AAA TG-3′; pGL3-Basic forward, 5′-AGA CCC ACG CTC ACC GGC TCC AGA-3′; pGL3-Basic reverse, 5′-ACG AGC GTG ACA CCA CGA TGC CTG T-3′. The amounts of the immunoprecipitated DNA were quantified by real-time PCR analysis using a LightCycler 480 (Roche) with the LightCycler 480 SYBR Green I Master reagent (Roche). W12 cells were cultured in an undifferentiated state as described [12], and the ChIP assay was performed without plasmid transfection.

Generation of nucleolin-knockdown cells and Western blotting. The stable nucleolin-knockdown cell line derived from HeLa cells was established by transfection of an expression plasmid for small hairpin RNA (shRNA) against nucleolin and subsequent selection of cells in the presence of 10 μg/ml puromycin. The shRNA-expression plasmid was constructed by cloning the shRNA target sequence for nucleolin (5′-GGA AGA CGG TGA AAT TGA T-3′) [13] into pBAsi-hU6 (Takara, Japan). For Western blot analysis, cell extracts were prepared by boiling cells in SDS-sample buffer. Protein samples were separated on a 12% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany), and probed with a specified primary antibody and a peroxidase-conjugated secondary antibody. Antibodies used were anti-nucleolin (MS-3, Santa Cruz, or 3G4B20, Active Motif), anti-PCNA (PC10, Santa Cruz), and anti-FLAG M2. Specific proteins were visualized using an ECL Western blot detection system (GE Healthcare).

Results

Identification of nucleolin as a protein binding to an HPV16 genomic fragment

Two regions in the HPV16 genome were chosen as targets to screen for cellular proteins that might potentially regulate transcription and/or replication of HPV (I and III in Fig. 1A). Fragment I (nt 7791–120) contains the regulatory sequence for the HPV16 early promoter P_{97} and the replication origin, whereas fragment III (nt 531–780) includes the late promoter P_{670}. To find specific binding proteins for these fragments, fragment II (nt 131–360) was used as a control for comparison, because no particular binding proteins were assigned to this region. These DNA fragments were generated by PCR so as to have 5′-biotin-labeled ends, coupled to streptavidin-conjugated magnetic beads and then incubated in a nuclear extract prepared from HeLa cells. After washing the beads, bound proteins were released and separated by SDS–PAGE, followed by silver staining. Among many protein bands detected, fragment III selectively bound to a 95-kDa protein (Fig. 1B), while no specific proteins were detected for fragment I. To identify the 95-kDa protein, the band was excised from the gel, digested with trypsin, then subjected to peptide mass fingerprinting. The list of observed mass fingerprints significantly fitted the predicted mass fingerprint of human nucleolin (p value = 0.0094). Western blot analysis with an anti-nucleolin antibody verified an enrichment of nucleolin in the bound fraction of fragment III (Fig. 1C).

In vitro binding of nucleolin to the HPV16 DNA

An electrophoretic mobility shift assay (EMSA) revealed that a recombinant nucleolin bound to a site in the HPV16 genome from nt 591 to 620. Human nucleolin from amino acid 289 to 710, which contains four RNA-binding domains and a C-terminal glycine/arginine-rich region (Fig. 2A), was expressed as a fusion protein with GST (GST–nucleolin) in bacteria and affinity purified (Fig. 2B). This truncated nucleolin has been shown to exhibit DNA-binding activity [6]. GST–nucleolin was examined in EMSA for its capability to form a complex with overlapping DNA probes having the HPV16 sequences from nt 531 to 660. Among the HPV16 probes tested, probe f generated a shifted band with GST–nucleolin (Fig. 2C).

Nucleolin has been previously reported to bind to a different location in the HPV18 genome *in vitro*, and the binding site has been assigned to the sequence 5′-TTGCTTGCATAA-3′ (nt 7642–7653 in the HPV18 genome) [14]. Similarity between the HPV18 sequence and probe f was explored, and the same sequence motif, 5′-TTGCXXXCAXA-3′, was found in the two sequences (Fig. 2D, upper panel). To test whether this sequence motif was recognized by nucleolin, base substitutions that have been shown to abolish the nucleolin binding to the HPV18 site were introduced into probe f. The mutations completely disrupted the GST–nucleolin binding to the probe (Fig. 2D), demonstrating that nucleolin bound to this motif in probe f in a sequence-dependent manner.

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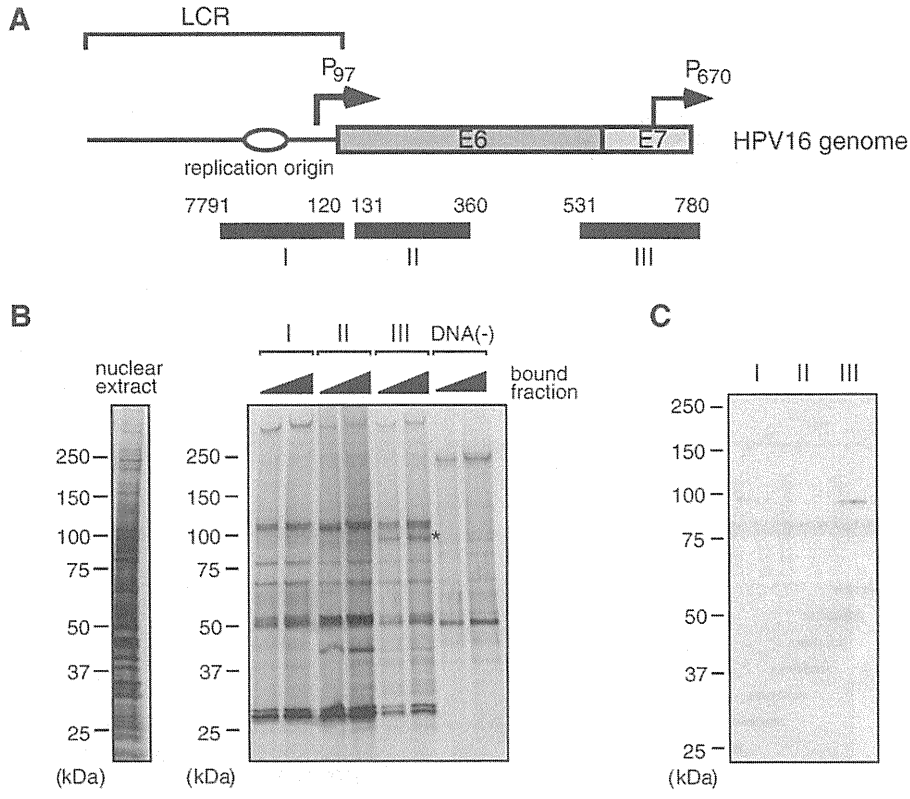

Fig. 1. Isolation of cellular proteins bound to HPV16 genomic fragments. (A) Schematic for locations of three HPV16 DNA fragments (I, II, and III) used to screen for binding proteins to HPV16 DNA. Numbers above the fragments indicate nucleotide positions in the HPV16 genome. The positions of the early promoter (P_{97}), the late promoter (P_{670}), the long control region (LCR), and the replication origin are presented. (B) The nuclear extract of HeLa cells was incubated with magnetic beads that were coupled with the HPV16 DNA fragments or with the beads alone. The bound fractions were recovered and resolved by SDS–PAGE, followed by silver staining. A 95-kDa band bound to fragment III is indicated by an asterisk. (C) Western blot analysis of the bound fractions using anti-nucleolin antibody (Santa Cruz).

GST–nucleolin bound to an HPV18 probe having the nucleolin-binding motif (Fig. 2D), which confirmed the integrity of our GST–nucleolin preparation. Additionally, several base substitutions were introduced into the motif in probe f (Fig. 2E, upper panel). All mutated probes gave rise to a band shift more efficiently than the original probe f (Fig. 2E), indicating that nucleolin recognizes this motif but it is not an optimal sequence for nucleolin binding.

Binding of nucleolin to the HPV16 DNA in cells

Chromatin immunoprecipitation (ChIP) analysis demonstrated that exogenous nucleolin bound to the HPV16 DNA in cells. An expression plasmid for FLAG-tagged nucleolin (FLAG-nucleolin) (Fig. 3A) was constructed and used for ChIP analysis. HeLa cells were transfected with the HPV16 reporter plasmid pGL3-P_{670}, which contains the HPV16 genomic region from nt 7003 to 868 [11], with or without the FLAG-nucleolin expression plasmid. In the presence of FLAG-nucleolin, an anti-FLAG antibody precipitated the HPV16 DNA fragment containing from nt 501 to 670 compared to the control IgG precipitate (Fig. 3B). Without FLAG-nucleolin expression, the anti-FLAG antibody did not precipitate the HPV16 DNA. The anti-FLAG antibody recovered an endogenous target of nucleolin, 18S rDNA, which indicated that exogenous FLAG-nucleolin behaved as endogenous one. The backbone plasmid lacking the HPV16 sequence was not precipitated with the anti-FLAG antibody in the presence of FLAG-nucleolin (Fig. 3C), demonstrating that the HPV16 sequence was responsible for the FLAG-nucleolin binding in cells. Similar results were obtained with human primary foreskin keratinocytes (data not shown), suggesting that the nucleolin binding to the HPV16 DNA is not specific to cancer cells.

Endogenous nucleolin also associated with the HPV16 DNA in cells. When the ChIP assay was performed in HeLa cells transfected with pGL3-P_{670} alone, the precipitate with an anti-nucleolin antibody showed an enrichment of the HPV16 DNA compared to basal level DNA obtained with a control antibody (Fig. 3D). To further examine binding properties of nucleolin to the HPV16 DNA in cells, a stable HeLa cell line expressing a reduced level of nucleolin was generated using an shRNA-mediated knockdown technique. Western blot analysis showed that the nucleolin level was reduced by one-third in the knockdown cells relative to parental cells (Fig. 3E). The ChIP assay revealed that the binding of nucleolin to the transfected HPV16 DNA was weakened in the knockdown cells compared to wild-type cells, and a similar reduction of nucleolin binding to rDNA loci was observed (Fig. 3F), suggesting that nucleolin's affinity for the HPV16 DNA is comparable to that for endogenous rDNA loci.

The ChIP assay was further extended to another human cell line, W12 cells, established from a cervical intraepithelial lesion and shown to maintain up to 1000 copies of the complete HPV16 genome as episomes in cell culture [12,15]. The immunoprecipitation with the anti-nucleolin antibody from the cross-linked chromatin of W12 cells enriched the HPV16 genomic DNA two to threefold compared to the control IgG precipitate, and a similar enrichment was observed with rDNA (Fig. 4). The results suggest that endogenous nucleolin is inherently bound to the HPV16 genome in the HPV16-infected cells.

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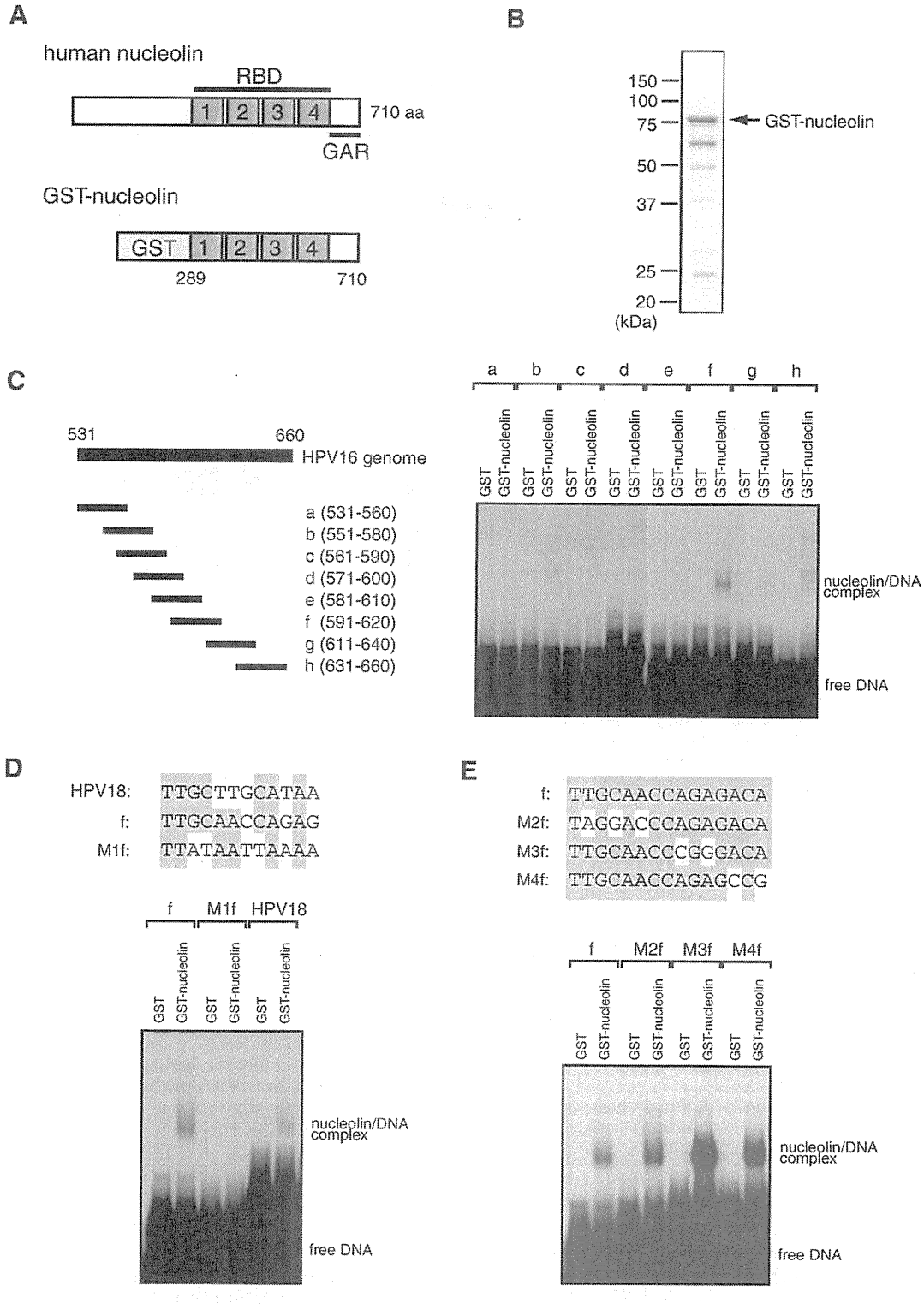

Fig. 2. *In vitro* binding of nucleolin to HPV16 DNA. (A) Schematic representation of human nucleolin and recombinant GST–nucleolin. Four RNA-binding domains (RBD1–4) and the glycine/arginine-rich region (GAR) are indicated. (B) SDS–PAGE analysis of GST–nucleolin with GelCode stain (Pierce). (C) EMSA showing a complex formation between GST–nucleolin and [^{32}P]-labeled oligonucleotide probes having the HPV16 genome sequence from nt 531 to 660. The genomic locations of the probes are presented on the left. (D) EMSA using mutated probe f to examine a sequence-specific binding of GST–nucleolin. Sequence alignment among the HPV18 nucleolin-binding sequence, probe f, and mutated probe f (M1f) is shown above. (E) Mutational analyses of probe f by EMSA. Base substitutions introduced into probe f are shown above.

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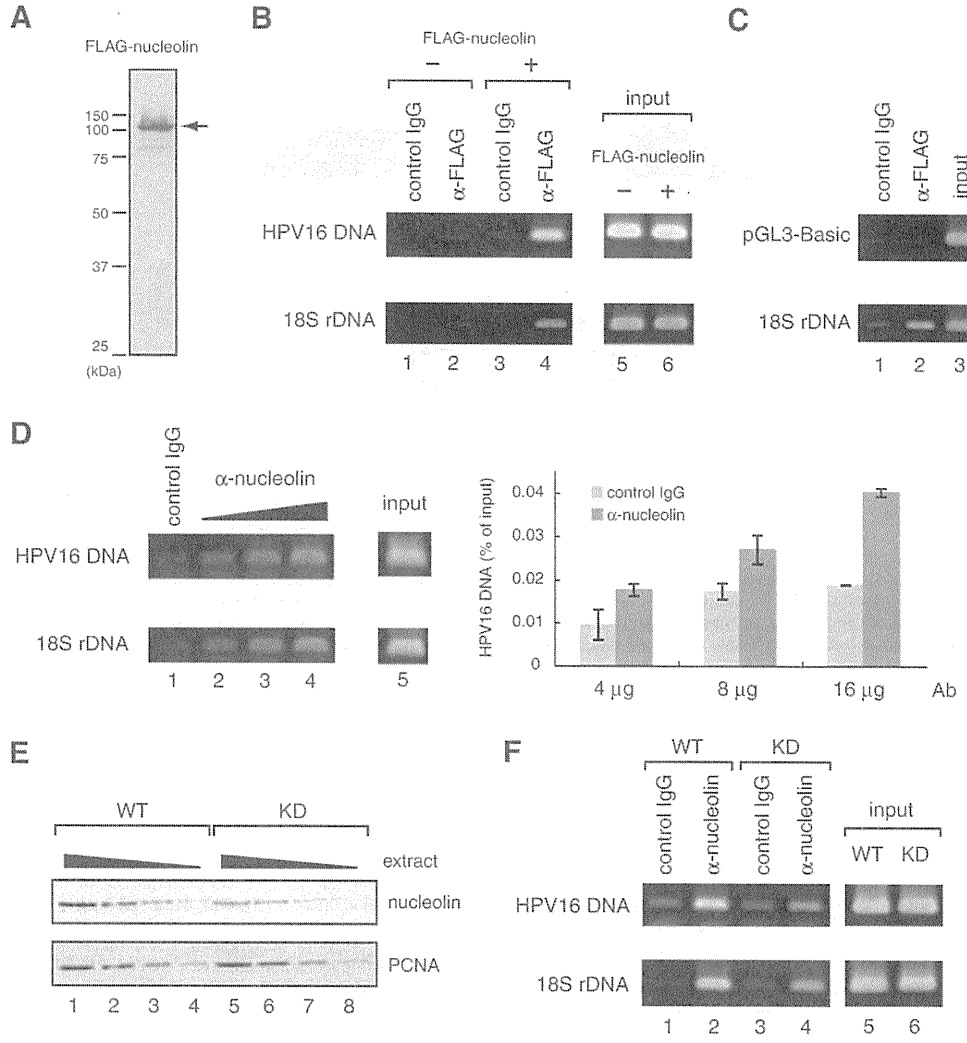

Fig. 3. Binding of nucleolin to HPV16 DNA in HeLa cells. (A) Western blot analysis of exogenous FLAG-nucleolin in HeLa cells using anti-FLAG antibody. (B) Chromatin immunoprecipitation analysis to detect binding of FLAG-nucleolin to HPV16 DNA in HeLa cells transfected with pGL3-P_{670}, which contains 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

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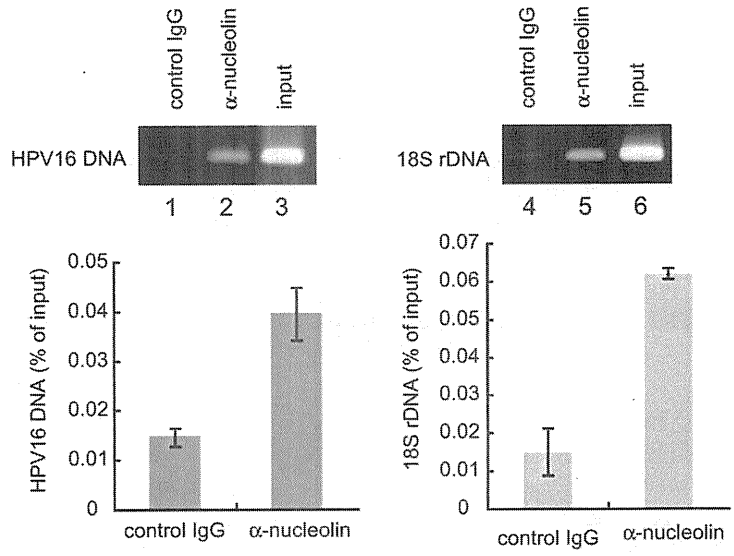

Fig. 4. Binding of nucleolin to HPV16 genome in W12 cells. ChIP analysis using W12 cervical neoplasia cells to detect binding of endogenous nucleolin to the HPV16 genome or rDNA. Cross-linked nucleolin/DNA complexes were immunoprecipitated with anti-nucleolin antibody (Abcam) (lanes 2 and 5) or control 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.

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

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

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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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