Defective 3A Trophoblast-Endometrial Cell Adhesion and Altered 3A Growth and Survival by Human Papillomavirus Type 16 Oncogenes

Hong You,¹ Yong Liu,¹ Martin J. Carey,² Curtis L. Lowery,¹ and Paul L. Hermonat¹

Departments of ¹Obstetrics and Gynecology and ²Emergency Medicine, University of Arkansas for Medical Sciences, Little Rock, AR

Abstract
Human papillomaviruses (HPVs) are found in trophoblasts of spontaneous abortions and replicate in these cells in culture. We used recombinant adeno-associated viruses (rAAV) to introduce the HPV-16 E6 and E7 oncogenes into 3A trophoblasts. AAV/E7/Neo-infected 3A trophoblasts died rapidly, but AAV/E6/Neo- and AAV/E6/E7/Neo-infected cells grew more rapidly than AAV/Neo-infected 3A cells and parental 3A. After G418 selection, the resulting E6-E7/3A and E6/3A cell lines were found to be highly defective for binding RL95 and HEC endometrial cells compared to Neo/3A and parental 3A. Serum requirements and soft agar colony formation analysis showed that E6-E7/3A had the most malignant phenotype, followed by E6/3A, with parental 3A cells having the lowest. E6/3A and E6-E7/3A were also immortal. Thus, HPV-16 oncogene expression may lead to outright trophoblast death, defective endometrial cell recognition, or a malignant phenotype. Any of these changes might lead to disruption/dysfunction of the trophoblast layer/gestational loss.

Introduction
The etiology of many miscarriages is unclear. However, human papillomaviruses (HPVs) are possibly one etiologic agent that needs to be investigated. There have been a number of clinical and molecular studies that indicate that HPV is associated with spontaneous abortions or that HPV commonly infects placental/fetal material (1–10). For example, Sikstrom et al. (1) found that women with a history of HPV infection had a higher risk of spontaneous abortion (odds ratio of 3). Malhomme et al. (2) have also found that 70% of spontaneous abortion specimens contain HPV by PCR analysis. Others have also found HPV-18 in 18% of hydatidiform moles and 50% of choriocarcinomas (malignant trophoblasts) (6). However, two groups find no evidence of HPV in such situations (11, 12).

An earlier study by our laboratory has found that HPV infection, as evidenced by the presence of HPV DNA, is 3-fold more prevalent in spontaneously aborted products of conception compared to elective abortions (60% versus 20%) (3). Furthermore, direct analysis of spontaneous abortion tissues by in situ PCR revealed that the HPV was preferentially infecting the trophoblasts within the chorionic villi of the placenta (3, 4). Recently, we have found that HPV-16 is able to replicate its DNA de novo and produce progeny in cultured placental trophoblasts (5). These later data were surprising as HPV was previously believed to be an exclusively keratinocyte/skin-specific virus. In trophoblasts, the HPV “early” genes (E6, E2) were expressed ahead of the “late” genes (L1), and infectious units of HPV appeared in a consistent manner with L1 expression (5). That HPV favors infection of trophoblasts and has full biological activity in these cells is noteworthy. Trophoblasts are critical cells of the placenta that are in direct contact with the maternal tissues, and serve multiple roles in the gestation. The disruption of the trophoblast layer, by HPV or any other infectious or chemical agent, might result in abnormal placentation, expulsion of the gestation, and even malignant disease (13).

If HPVs are verified to be significant factors in spontaneous abortion, it seems likely that the E6 and E7 oncoproteins of HPV would have significant roles in this pathology. E6 and E7 are significant factors in the development of cervical cancer and cooperate in immortalization of primary keratinocytes in tissue culture (14). Much is known about the biochemistry of these oncoproteins. The E6 protein binds to p53, promoting its degradation via ubiquitin pathways of proteolysis (15, 16). E7 binds to the retinoblastoma protein (Rb), and activates genes promoting DNA synthesis and cell proliferation by interaction with the hypophosphorylated form of Rb, releasing active E2F or AP-1 transcription factors (17, 18). However, increased activity of E2F-1 may also sensitize cells to apoptosis, a response that can be inhibited by E6 in keratinocytes (19).

Because of the evidence showing that HPV may be associated with some spontaneous abortions, it is important to understand the potential effects of its genomic components on trophoblast physiology. To elucidate the effects of these two important oncoproteins, E6 and E7, on trophoblasts, the present study investigates HPV-16 E6 and E7, alone and in combination, on trophoblasts in tissue culture. The results indicate that the effects of E6 and E7 on trophoblasts mimic, to a large extent, their effects on keratinocytes, particularly regarding oncogenically related changes. However, an unexpected effect on trophoblast-endometrial cell adhesion was also observed.
Results

Alteration of 3A Trophoblast Growth by E6 and E7

3A trophoblasts were chosen as the experimental cell type because they are well characterized and represent a near-normal trophoblast. The genomic structures of the viruses used in this study are shown in Fig. 1A, as is the titering of the virus stocks in Fig. 1B. To assess the affects of E6 and E7, low-confluence cultures of 3A trophoblasts cells were infected with recombinant adeno-associated virus (rAAV)/E6/Neo, AAV/E7/Neo, and AAV/E6-E7/Neo viruses and the cultures were observed over time. AAV/Neo virus infection was used as a control. At least two effects from the infections were observed. First, after infection, the E6/3A and E6-E7/3A grew faster and generated a subset of larger colonies than the Neo/3A and parental cells. This is shown in Fig. 2A. Second, the E7/3A became round shaped and began to detach from the plate after 3 days of infection. These cells were predominantly dead on day 4 (Fig. 2, A and B) as judged by trypan blue exclusion. Fig. 2B shows the growth rate of the infected and uninfected cultures, and highlights the faster growth rate of the E6/3A, followed by E6-E7/3A. The AAV/Neo vector-infected cells were similar to the 3A cells.

Altered 3A Trophoblasts Contain Transduced DNA and Express E6 and E7

The surviving E6/3A, E6-E7/3A, and Neo/3A cultures were then selected with 100 μg/ml G418 to obtain pure populations of transduced cells. After 20 days of selection, the control 3A cells were dead, and the E6/3A, E6-E7/3A, and Neo/3A that were stably transduced formed large G418-resistant colonies. Each infection resulted in approximately 20–40 such colonies. Each of the cultures was then trypsinized, and replated to generate a multiclonal mixed population culture. These cells were analyzed for the presence of the HPV oncogene DNA by dot blot hybridization analysis of total cellular DNA, probing with 32P-E6 DNA. Both the G418-selected E6/3A and E6-E7/3A cultures contained the oncogene sequences (data not shown).

The cells were next analyzed for expression of the HPV transgenes. Polyadenylate selected RNA was isolated from the various cell populations and analyzed for the presence of E6 and E7 transcripts. TFII B RNA, a cellular housekeeping transcription factor, was also analyzed as a control. Reverse transcription-PCR (RT-PCR) analysis, shown in Fig. 3, revealed that E6/3A and E6-E7/3A expressed the expected oncogenes. E6/3A demonstrated only E6 mRNA, whereas E6-E7/3A demonstrated both E6 and E7 mRNA. Parental 3A cells expressed only TFII B, and expression of this gene appeared to be relatively stable in all cell lines. Finally, mRNA samples were also analyzed by direct PCR and failed to generate oncogene products.

HPV-16 Oncogenes Altered 3A Trophoblast Adhesion to RL95 and HEC Endometrial Cells

A central characteristic and function of the trophoblast is to bind endometrial cells. This is needed for both implantation and placental maintenance. To analyze this central characteristic, a heterologous cell-cell adhesion assay was used, which involved the binding of 51Cr-labeled, single cell trophoblasts in suspension to confluent adherent-monolayer RL95 and HEC endometrial cells. It was shown (Fig. 4A) that after 2 h of incubation, E6/3A and E6-E7/3A displayed much weaker binding than the parental 3A or Neo/3A cells. This highly defective binding was also observed with a second endometrial cell line tested, HEC (Fig. 4B). Representative pictures of the HEC-based experiments are shown in Fig. 4C. As can be seen, clusters of cells are observed bound on top of the HEC cell monolayer when 3A or Neo/3A cells were added in coculture. Few such clusters were observed with the addition of E6/3A or E6-E7/3A cells.

Trophoblast Growth Characteristics Changed After G418 Selection

The now “pure” transduced and G418-selected cells were then remeasured for altered growth characteristics. Surprisingly, after selection, the growth rate of the E6-E7/3A cells was much faster than E6/3A, with Neo/3A and 3A parental cells both lagging behind (Fig. 5A). This difference could be seen by

FIGURE 1. Structure and titering of virus. A, Genomic structure of the AAV/Neo, AAV/E6/Neo, AAV/E7/Neo, and AAV/E6-E7/Neo viruses. B, Titering of the virus stocks by analyzing the amount of DNA encapsidated within virus particles within a volume of virus stock.
comparing Fig. 5A with Fig. 2B (before G418). Differences could also be observed on the level of cellular morphology (Fig. 5B). The parental 3A and Neo/3A cells contained a mixture of cytotrophoblast-like cells (small, mononuclear cells) and larger syncytiotrophoblast-like cells (large, multinucleated cells). The E6/3A contained more cytotrophoblasts and fewer syncytiotrophoblasts. In contrast, the E6-E7/3A cells were almost exclusively small, hyperchromatic, densely growing cells with very few syncytiotrophoblasts. These appearances, going from a normal to a more malignant phenotype, would appear to be consistent with the growth characteristics after G418 selection. Human chorionic gonadotropin (HCG) secretion was also measured as an indicator of trophoblast differentiation. HCG levels secreted by the E6/3A and E6-E7/3A were not detectable as measured by ELISA assay, whereas the parental cell secreted HCG (14.0 ± 3.9 IU/ml).

Alteration of 3A Trophoblast Serum Dependence and Growth in Soft Agar

One generalized malignant characteristic is a lowered dependence on serum for growth. To determine if this might be the case for the genetically altered 3A cells, different concentrations of fetal serum, from 7% to 0%, were used in their DMEM. The altered and G418 selected, along with parental 3A cells, were grown over 12 days and the results are shown in Fig. 6. As shown, both E6/3A and E6-E7/3A had lower serum dependence, with E6-E7/3A being almost serum independent. Thus, the presence of the two oncogenes appears to induce a more malignant phenotype than E6 alone.

Another generalized malignant characteristic is the ability for cells to form suspended colonies without an attached surface. To determine if the HPV oncoproteins could contribute to this phenotype, the G418-selected populations, along with parental 3A cells, were suspended in soft agar. Fig. 7, A and B, shows that the E6/3A and E6-E7/3A cells demonstrate more malignant characteristics, with increased ability to grow in soft agar over that of the parent. It should be noted that the E6-E7/3A cells grew much faster and formed more colonies than E6/3A or parental 3A cells (Fig. 7B, \( P < 0.05 \)).

Immortalization of HPV-16 Oncogenes Altered 3A Trophoblast

E6/3A, E6-E7/3A, and parental 3A were also continuously grown and passed to determine immortalization. Both the E6/3A and E6-E7/3A cell lines were able to survive for over 6 months. We consider these cells to be immortalized. In contrast, the growth rate of the parental 3A cells declined at 4 months, and they were fully senescent at 6 months.

Discussion

In previous in vitro studies, we have shown that HPV-16 can replicate and produce progeny virus in cultured placental...
trophoblasts (5). Additional data demonstrated that HPVs are preferentially found in the cells of the trophoblast layer of spontaneous abortions. These data strongly suggested that HPVs can infect placental trophoblasts and are associated with spontaneous abortion. Possibly, the most noteworthy genes encoded by HPV are the oncogenes E6 and E7. They are well known to be the etiologic basis for a number of related pathologies in keratinocytes/skin, including condyloma, dysplasia, and malignancy (21). The current investigation demonstrates that multiple physiological changes result from the introduction of E6 and E7 into 3A trophoblasts. These changes include highly defective endometrial cell recognition, cellular death, and induction of a malignant phenotype. The latter two changes have been described before in keratinocytes and were not unexpected (22–24). Weak cellular binding is a new phenotype attributable to these genes and would seem to be central to the issue of spontaneous abortion. However, any of the changes identified in this study might contribute to gestational pathology.

The 3A-RL95 or 3A-HEC cell adhesion assay, using representative trophoblastic and uterine epithelial cells, may mimic the initial steps in embryo implantation. The observed decrease in adhesion that we have observed to be caused by the introduction of E6 and E7 might suggest the potential for abnormal implantation or expulsion of the early embryo. However, these changes in adhesion may be significant beyond implantation as well. This assay might additionally serve as a model for longer term placental-endometrial attachment. The HPV oncogenes caused a strong alteration in the affinity between the two cell types. Any weakening in the cellular attachments between the placenta and endometrium can only be viewed as a potential pathology.

Another significant physiological change was the rapid death caused by AAV/E7/Neo infection. Thus, these cells did not survive to be tested in the endometrial adhesion assay. The rapid death suggests a form of E7-induced apoptosis. A similar result has been reported in cervical keratinocytes in which high-level expression of E7 sensitized cells to apoptosis (24). In any case, this pathology (cell death) must be added to the defective endometrial cell binding seen by the surviving HPV oncogene-positive cells, all suggesting that the presence of HPV in...
trophoblasts can only be trouble for the gestation. Future research will address if this cell death is a type of apoptosis, and preliminary data are consistent with hypothesis (DNA ladders are seen). We are unsure as to what increased malignant characteristics of trophoblasts might mean for a gestation. Normal trophoblasts are already considered to have some malignant characteristics. In human gestations, these characteristics are necessary for successful placentation. However, increases in these malignant characteristics are likely not a normal or well-tolerated physiological situation. E6 and E7 are pathologically important HPV genes and their oncogenicity is due in part to their capacity to inactivate the cellular tumor suppressor genes, P53 and Rb, respectively. These two oncogenes are believed to have vital functions in the development of HPV-associated cervical cancer (22, 23). Coexpression of E6 and E7 was even more effective than E6 alone in trophoblasts. In trophoblasts, E6/E7 may induce or promote a dysplastic/carcinogenic pathway that might ultimately result in molar pregnancies or choriocarcinoma. A final possible HPV-associated pathological mechanism could be E6 and E7’s effects on the immune system. For example, new antigenic epitopes (e.g., E6 and E7) might be expressed and recognized (20). The resultant host response could result in expulsion of the embryo and/or abnormal placenta.

In summary, HPV oncogenic component genes E6 and E7 have significant effects upon the general cellular characteristics of trophoblasts, including cell survival, endometrial cell binding, proliferation, differentiation, and immortalization. It might be considered that any of these changes in the trophoblasts might be responsible for altered trophoblast/placental physiology and could contribute to spontaneous abortions. Thus, these data support our hypothesis that HPV may be associated with some spontaneous abortions and further suggest possible etiologic mechanisms. Finally, the HPV-E6- and E6-E7-altered 3A trophoblasts may provide new reagents and opportunities for the study of antiviral intervention for HPV-infected gestations in vitro.

Materials and Methods

Cell and Plasmids

3A trophoblasts (ATCC CR-1583) were grown at 37–39°C in DMEM with 7% fetal bovine serum (FBS), except where otherwise indicated. These cells have normal trophoblast traits (e.g., synthesize HCG and alkaline phosphatase) at this temperature (25). The 3A cultures were also heterogeneous in nature, containing both cytotrophoblasts (small precursor cells) and syncytiotrophoblasts (large, mature, multinucleated cells). RL95 (ATCC CRL-1671) and HEC (ATCC HTB-112) were uterus endometrium carcinoma cell lines; they were grown in DMEM with 10% FBS. AAV/HPV-16 E6, E7, and E6-E7/Neo genomes were constructed in a similar strategy as previously described (26). Briefly, the wild-type AAV genome pSM620 was partially digested with BsaI to delete the internal AAV sequences from map units 6–95 (nucleotides 286–4460), and a specially designed polylinker was ligated in place, resulting in the AAV vector plasmid Δ6-95/PL1. Into this polylinker, the neomycin (Neo) resistance gene and HPV-16 E6, E7, and E6-E7 open reading frames, which were cloned by PCR amplification, were subsequently ligated (Fig. 1A).

Preparation of rAAV Virus Stocks and Transduction of 3A Cells

The AAV/E6/Neo, AAV/E7/Neo, AAV/E6-E7/Neo, and AAV/Neo virus stocks were made from 293 cells by transfecting 293 cells with 5 μg of the AAV vector along with 5 μg of the AAV/Ad complementor plasmid pSH3 (27). The copies of each virus stock were 1 × 107 encapsidated genomes (Fig. 1B).

The 3A trophoblasts were plated at an approximate density of 1000 cells/35 mm culture plate in DMEM with 7% serum; these cells were infected with the rAAV virus containing E6, E7, E6-E7, or Neo at the multiplicity of infection of 1000 encapsidated genomes. After incubation for 4 h, the cells were trypsinized and replated. After 12 days, duplicated dishes were fixed by

![FIGURE 6. Serum growth requirements of E6-E7/3A and E6/3A after G418 selection. Different concentrations of fetal serum from 7% to 0% were used in DMEM; the altered and G418 selected, along with parental 3A cells, were grown in 35-mm plates over 12 days and stained with methylene blue. Note that both E6/3A and E6-E7/3A had lower serum dependence, with E6-E7/3A being almost serum independent.](image-url)

![FIGURE 7. Colony-forming abilities of E6-E7/3A and E6/3A by soft agar assay. Both the E6/3A and E6-E7/3A cells demonstrate the increased ability to grow in soft agar over that of parental 3As. However, the E6-E7/3A cells produced significantly more colonies than E6/3A or parental cells. A, Representative plates. B, Quantitative measurements of resulting colonies.](image-url)
formalin and stained with methylene blue. Other equivalent dishes were continuously grown under 100 μg G418/ml selection for 20 days. Thereafter, all the G418-resistant clones (20–40 for each infection) were grown together as a mixed population, and used for the following experiments. The 3A cells infected with AAV/E6/Neo, AAV/E7/Neo, AAV/E6-E7/Neo, or AAV/Neo viruses are thereafter described as E6/3A, E7/3A, E6-E7/3A, or Neo/3A in the following text, respectively.

**Dot Blot Analysis**

For Dot blot analysis of E6/3A, E6-E7/3A, and parental 3A cells, certain amounts of the total cellular DNA from each experimental cell group were dot blotted as described previously (28) and probed with 32P-labeled HPV-16 E6 DNA.

**Messenger RNA Isolation and RT-PCR**

HPV-16 oncogenes-infected 3A trophoblast mRNA expression was measured by RT-PCR along with a cellular mRNA control. Total RNA was isolated at the indicated days using Trizol reagent (Life Technologies, Inc., Rockville, MD), according to the manufacturer’s protocol, and treated with 5 units/μg of RNase-free DNase I (Promega Co., Madison, WI) at 37°C for 2 h. Messenger RNA was then separated using the Oligotex mRNA Mini Kit (Qiagen, Inc., Valencia, CA) according to the supplier’s instruction. The first-strand cDNA synthesis was performed at 37°C for 1 h in a final volume of 25 μl reaction buffer [1 μg mRNA; 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl2; 10 mM DTT; 0.5 μg oligo(dT)15 (Promega Co.); 0.5 mM of each of the four dNTPs; 30 units of RNasin (Promega Co.); and 200 units of M-MLV Reverse Transcriptase RNase H Minus (Promega Co.)]. PCR amplification of the cDNA was performed in a 100-μl reaction volume which contained 2.5 units Taq DNA polymerase (Fisher Scientific Co., Pittsburgh, PA); 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2 mM MgCl2; 0.2 mM of each of the four dNTPs; 1 μM of each upstream and downstream primers specific for the cDNA template; and 10 μl cDNA template.

**Trophoblast-RL95 Endometrial Cell Binding Assay**

This experiment was undertaken similar to that described previously (30). First, suspensions of E6/3A, E6-E7/3A, Neo/3A, or parental 3A cells (2 × 105 cells/ml) were labeled by adding 50 μCi of 35Cr and incubated for 60 min at 37°C. The labeled cells were then washed three times with PBS. Next, cultures of confluent RL95 or HEC cells were prepared in a 24-well plate. The 35Cr-cells were then added to the medium covering the endometrial cells and incubated for 2 h at 37°C. At that time, the medium and nonadherent cells were removed and the monolayer was gently washed three times with PBS. The labeled 3A trophoblasts that remained bound to the RL95 or HEC cells were then extracted by the addition of 0.5 M NaOH and 1% SDS, and measured by liquid scintillation counting.

**HCG in 3A Trophoblast by ELISA**

3A trophoblast cells were plated at 1 × 106/ml and cultured in DMEM with 7% FCS. After incubation for 48 h at 37°C, the supernatant was removed and detected in duplicate by a commercially available ELISA kit (ICN Pharmaceuticals, Orangeburg, NY) utilizing HCG standards ranging from 0 to 300 mIU/ml. The minimum detectable concentration of HCG was 2.0 mIU/ml. This assay was performed according to the manufacturer’s instructions.

**Analysis of Cell Growth Kinetics and Serum Requirements**

The E6/3A, E6-E7/3A, and Neo/3A cells were compared with the parental cells for their ability to grow and serum requirements. Every 4 days, the cells, in triplicate, were trypsinized and counted to provide number for determination of growth kinetics. A total of 5 × 103 cells of each type was plated in 35-mm plates in DMEM plus 7%, 1.5%, 0.25%, and 0% FBS. After 12 days, equivalent cultures resulting from different concentration serum were fixed with formaldehyde (4%) and stained with methylene blue.

**Soft Agar Growth Assay**

The E6/3A, E6-E7/3A, and parental 3A cells were plated in double-layered agar, in triplicate, to determine the frequency of soft agar colony formation in the cell population (29). Briefly, 2 ml of 42°C, 0.4% soft agarose/complete culture medium were added to 5 × 104 HPV-altered 3A trophoblasts and parental cells, gently pipetted up and down to mix and put onto 35-mm dishes with a 0.8% soft agarose underlay. The agarose was allowed to harden for 10 min and then incubated at 37°C, 5% CO2 for 14 days. The number of colonies larger than 0.5 mm diameter was then counted using an inverted microscope.

**Immortalization Assay**

The transformed cells were continuously fed and passaged, splitting 1–5 when near confluence. The passage at which cell growth stopped was monitored. Continued cell growth past 6 months indicated immortalization.

**Statistics**

All results are expressed as mean ± SD. The data were analyzed by nonparametric ANOVA test. If differences were detected between means, Newman-Keuls test was used for multiple comparison. Differences were considered as significant if P < 0.05.

**References**


Defective 3A Trophoblast-Endometrial Cell Adhesion and Altered 3A Growth and Survival by Human Papillomavirus Type 16 Oncogenes

March of Dimer Grant 6-FY99-188 (to P.L.H.).

Hong You, Yong Liu, Martin J. Carey, et al.


Updated version
Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/1/1/25

Cited articles
This article cites 29 articles, 7 of which you can access for free at:
http://mcr.aacrjournals.org/content/1/1/25.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/1/1/25.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.