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MATERNAL-FETAL MEDICINE

HPV-16 exposed mouse embryos: a potential model for pregnancy wastage

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Abstract
Purpose Placentas from spontaneous abortions and preterm deliveries have a higher prevalence of Human papillomavirus (HPV) compared to placentas from elective abortions and term births. The objective was to determine the effects of HPV-16 on the adhesion and implantation properties of early embryo trophoblasts.
Methods Two-cell mouse embryos were cultured (medium G2, 5 % CO_2, 37 °C) for 72–96 h and exposed to either HPV-16 rich SiHa cell lysates which were refrigerated after mechanical lysis, thawed lysates which had been frozen for freeze/thaw lysis method, or control medium, incubated (4–5 days) and evaluated by microscopy ($N = 96$ embryos, 3 repeated experiments). Trophoblasts were stained and images were digitized. Adhesion and dimension data were analyzed by Chi-square and *t* test, respectively.
Results HPV-16 exposed embryos exhibited less adhesion through reduced implantation compared with the control (combined lysates 53.8 vs. 85.7 %, $P < 0.05$). Refrigerated and thawed lysate groups had similar reduced implantations (58.3 vs. 50.0 %). Of the embryos with implantation, 100 % in the refrigerated lysates were noted to have loose or abnormal adhesion. This was measured when embryos were noted to be lost after washes with HTF. There was no difference in trophoblast viability among the groups. Total trophoblast area was greater in the HPV-16 exposed frozen lysate group (1,881.8 ± 605.3 vs. control 848.8 ± 298.0 square units, mean ± SEM).
Conclusions HPV-16 inhibited trophoblasts adhesion needed for normal implantation, but not embryo development. Total trophoblast spread was increased after HPV-16 exposure suggesting that HPV-16 altered trophoblast migration. These results suggest that HPV-16 may induce abnormal placental growth resulting in pregnancy wastage.

Keywords HPV · Trophoblasts · Mouse · Placenta · Miscarriage

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Introduction

Human papillomaviruses (HPVs) are highly prevalent small double-stranded DNA viruses with a known tropism for epithelial cells. Persistent infections with high-risk HPV serotypes are recognized as the causative agents in nearly all cervical cancers. HPV types 16 and 18 are responsible for causing 70 % of all cases [1]. The National Cancer Institute's Surveillance, Epidemiology and End Results (*SEER*) program estimates that approximately 12,170 women will be diagnosed with and 4,220 women will die of cervical cancer in 2012 [2]. Although the role of HPV as an infectious etiology of cancer has been extensively studied, the discovery of HPV in the placental cells has only been recently established.

Various studies have reported that various HPV types can infect human placenta during pregnancy and replicate in the placental trophoblast cells [3–7]. This led to the investigation of the role of HPV in spontaneous abortions. The infection rates in spontaneous abortions of first and second trimester are reported to be as high as 50–70 % [4–6, 8]. HPV has been recovered more frequently in placentas obtained from patients with spontaneous preterm labor and spontaneous abortions than from term or elective

abortions [4, 9]. More recently, HPV was identified in 47.2 % placentas obtained during the birthing process in a population of Mexican women [10]. The high percentage of HPV infection in the placenta is a concern in light of known pathogenic effects of HPV and its role in the etiology of diseases [1, 2].

These poor early pregnancy outcomes are may be due to effects of HPV on trophoblast development and implantation. HPV-16 oncoprotein expression in trophoblastic cell lines have been associated with changes in viability, reduced adhesion, enhanced migration and invasiveness of the transformed trophoblast cells [3]. Early studies by Brackmann et al. [11] showed that HPV had the capacity to transform cells derived from mice. This study investigated the role of HPV-16 in the transformational changes of mouse embryo inner cell mass and trophoblast cells which lead to gestational trophoblastic disease (GTD). Interestingly, the trophoblast cells share similar properties with malignant tumor cells such as metabolic, invasive and migratory capacities [3, 12, 13]. The objective of this study was to determine the effects of HPV-16 on early embryonic development.

Materials and methods

HPV-16 lysate preparation

SiHa cells derived from human cervical squamous cell carcinoma (ATCC, American Type Culture Collection, Manassas, VA, USA) were previously reported to each contain 1–2 copies of HPV-16 integrated at chromosome 13q21-31 [14]. To prepare the lysate, the SiHa cells were cultured in eagle minimal essential medium (MEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (Invitrogen), penicillin (100 U/ml, Sigma Chemical Co., St. Louis, MO, USA) and streptomycin (100 μg/ml, Sigma Chemical Co). The cells were passaged and used at approximately 80 % confluence and stored in the refrigerator (4 °C) for one day prior to use [15]. The SiHa cells were equally divided into two tubes and centrifuged at 1,000 rpm for 10 min. The supernatants were decanted and the pellet combined in a microfuge tube containing 0.4 ml of G-2 plus version 5 medium (G-2 v5, VitroLife, Englewood, CO, USA). Optimal cell lysis was achieved by both mechanical and freeze–thaw methods. The cells were lysed using a sterile glass rod to release the HPV-16 gene fragments. The cell extract was then divided into two vials, one was stored in the refrigerator at 4 °C until later use and the other was frozen at −23 °C for up to a week to lyse the cell further prior to addition to the embryos. The lysates were thawed to 37 °C prior to adding them to the mouse embryo cultures to prevent cold shock effects on the developing embryo.

Culture of two-cell mouse embryo to the implantation stage

Cryopreserved two-cell mouse embryos in cryo straws were obtained from a commercial source and stored in liquid nitrogen until use. The straws were thawed and the contents expelled into a droplet of G-2 v5 medium on a petri dish. Exclusion criteria included darkened embryos, embryos with only one viable blastomere and lysed embryos that did not survive cryopreservation. The embryos were washed twice more in G-2 v5 medium. The washed embryos were pipetted into an eight-chamber tissue culture slide (Nunc Lab-Tek, Sigma-Aldrich, St. Louis, MO, USA) containing 0.4 ml of G-2 v5 medium. The tissue culture slides were placed in a humidified large petri dish and incubated at 37 °C, 5 % CO_2-in-air mixture. After 3 days of incubation, the embryos were randomly divided into 3 treatment groups: control, refrigerated SiHa or frozen SiHa. The refrigerated and frozen HPV SiHa cell lysates were warmed at 37 °C and 10 μl of each cell lysate was pipetted into the respective treatment group. The G-2 v5 culture medium was added to the control group. The chambered slides were returned to the 37 °C incubator and evaluated after 5 additional days of incubation. The experiment was repeated five times and data pooled for each respective group.

Embryo assessment

Assessment of embryo cell stage was carried out by visual grading using phase contrast in an inverted microscope (Nikon Instruments, Melville, NY, USA). Embryos were classified according to the final observed cell stage: two-cell, morula, blastocyst, hatching and implanting stages, as well as a category for apoptosis and necrosis. The number of developed embryos progressing beyond the two-cell stage in each treatment group was determined and compared with the control group. The percentage of implanted embryos was defined as the number of hatched blastocysts that implanted on the surface of the slide tissue culture matrix. Adherence was defined as embryos that remained securely adhered after rinsing with human tubal fluid culture medium (HTF, Irvine Scientific, Santa Ana, CA, USA). The percentages of embryos with loose adhesion lost through rinsing were determined and presented in Table 1.

Dual fluorescence stain analysis

The dual fluorescence stain method [16] was used to distinguish apoptotic or necrotic embryos from the viable

Table 1 Influence of HPV-16 SiHa cell lysates (refrigerated 4 °C vs. frozen −23 °C) on the development of two-cell mouse embryos in vitro for 8 days at 37 °C and 5 % CO_2-in-air mixture

Treatment	Number of developed embryos per total (%)	Number of implanted per developed embryos (%)	Number of embryos with loose adhesion (%)	Number of trophoblast with viable nucleus (%)
Control	14/20 (70.0)	12/14 (85.7)	9/12 (75.0)	145/186 (78.0)
Refrigerated lysate	12/26 (46.2)	7/12 (58.3)	7/7 (100.0)	43/48 (89.6)
Frozen lysate	14/26 (53.8)	7/14 (50.0)*	5/7 (71.4)	136/169 (80.5)
Combined lysates	26/52 (50.0)	14/26 (53.8)*	12/14 (85.7)	179/217 (82.5)

* Different from control $P < 0.05$

embryos. One drop of bisbenzimide (5 µl of 10 µM, Hoechst 33342, Sigma Chemical Co., St. Louis, MO, USA) was added into each chamber of the tissue culture slides containing the implanted embryos. After 2 min, 5 µl of propidium iodide (32 µM, Sigma Chemical Co., St. Louis, MO, USA dissolved in saline) was added. After a minute, the culture medium was discarded and the implanted embryos were washed with pre-warmed HTF medium. The chamber walls were removed using the peeler tool included in the Lab Tek II eight-Chamber Slide kit. A cover slip was placed over each slide and the embryo status was assessed using an epifluorescence UV-microscope set at magnification of 500× (Nikon Optiphot, Nikon Instruments, Melville, NY, USA). The percentages of viable, apoptotic and necrotic embryos were determined and the fluorescent images were digitized and recorded. Viability was defined as the ability of the embryo to exclude fluorescent dye, while apoptotic and necrotic embryos were identified by their ability to take up bisbenzimide or propidium iodide stain, respectively.

Embryo morphology stain

Trophoblast cells and inner cell mass nuclei and cytoplasm were stained using the Spermac (Stain Enterprises Inc., Republic of South Africa) stain procedure. The slides were rinsed in HTF medium and the cover slip was gently removed. Excess HTF was removed from each slide and the implanted embryos were fixed using the 4 % formalin fixative provided in the Spermac kit (5 min). The fixative was rinsed off with water and each slide dipped into Spermac stain A (Rose Bengal mixture) for 2 min, followed by stain B (Pyronin Y mixture) for 1 min and stain C (Janus Green mixture) for another minute. The slides were rinsed in water, air-dried and stored in a dark drawer until the time of analyses.

Spectrophotodensitometry

Cell dimensions were measured using a spectrophotodensitometric method which facilitated the determination of embryo growth and migration. The Spermac-stained inner cell mass and trophoblast cells were located on each slide using the Nikon Diaphot inverted microscope (400× magnification) and the images were digitized and recorded. The pre-analytical phase included using Corel Paint Shop Pro Photo ×2 software to standardize each image. An outline of the total implanted embryo was traced and a calibrating grid overlay was added to the selected image. The area of each inner cell mass or trophoblast nucleus as well as the area of each implanted embryo was calculated from the grids and data were recorded on Microsoft Excel spreadsheets for analysis. Digitized processed images involved relative magnification. For accuracy, surface area measurements were expressed in arbitrary square units.

Statistical analysis

Comparative analysis utilizing Chi-square and Student's *t* test was performed using Epistat Services (Version 2.1, Richardson, TX). A value of $P < 0.05$ was considered significant.

Results

Implantation

Embryos exposed to both the refrigerated and frozen HPV-16 lysates demonstrated 37.2 % less implantation when compared to control (Table 1). The hatched blastocysts in each of the refrigerated and frozen HPV-16 lysate groups failed to implant to the surface of the culture slide plastic matrix. These hatched blastocysts remained compacted in spherical structures. In contrast, control hatched blastocyst cells adhered to the surface of the culture slide plastic matrix and formed a visibly discernable inner cell mass and trophoblast cells layers. The percentages of embryos developing beyond the two-cell stage were similar in the HPV-16 groups and the control group.

Adhesion

Implanted embryos exposed to HPV-16 in the refrigerated group had 100 % inhibition of secure adhesion. The

percentage did not reach significance due to the low number of implanted embryos in this group. Additionally, there was no statistical difference in the adhesion of the implanted trophoblasts between the refrigerated versus frozen HPV-16 lysate groups. Interestingly, however, dual-stain fluorescence microscopy analyses revealed no difference in the viability of the trophoblast cells among the treatment groups versus the control.

Migration and viability

The migratory or invasive activity measured in terms of the mean area of each trophoblast cell determined through spectrophotodensitometry showed a significant difference between the HPV-16 frozen lysate group and the control (1,881.8 ± 605.3 vs. 848.8 ± 298.0 arbitrary square units, mean ± SEM, respectively). There was no measurable data for the refrigerated group due to complete embryonic loss from total inhibition of adhesion in this group.

Discussion

Clinical evidence suggests that HPV infection is associated with early pregnancy loss. HPV infection rates in spontaneous abortions of first and second trimesters have been shown to be in the 50–70 % range [4–7]. Moreover, placentas from spontaneous abortions and preterm deliveries have been linked to a higher prevalence of HPV infection compared with placentas from elective abortions and term births [4]. The results propose a possible mechanism for HPV associated early pregnancy loss. This study demonstrated that HPV-16 interfered with the implantation processes which resulted in embryonic loss. In addition, there was no difference between the refrigerated HPV-16 lysate and frozen lysate groups suggesting that the virulence of HPV-16 was maintained at both storage temperatures. The HPV-16 exposed embryonic cells at the hatched blastocysts stage were viable, but remained aggregated in spheres. In contrast, the control embryo attached and formed a flattened layer of trophoblast cells with giant nuclei and a central core of smaller cells forming the inner cell mass.

Altered trophoblast invasiveness was also found after exposure to HPV-16 [17]. The mean surface area dimension of each trophoblast cell exposed to HPV-16 was over twice that of the control trophoblast cell. Furthermore, the pseudopod structures spread out in random migratory directions and in segments, which may be due to the loss of normal regulation process found in normal pregnancy. These results are consistent with findings from other investigators. Impaired cell adhesion in trophoblast cells or in BeWo ('trophoblastic-like cells') choriocarcinoma cell lines is linked to the expression of HPV-16 early oncoproteins E5, E6 and E7 [3, 18, 19]. All 3 oncoproteins are shown to decrease trophoblast cell adhesion in endometrial cells and inhibit the implantation process [19–21]. HPV-16 oncoprotein expression in cervical and trophoblastic cell lines has been associated with the changes in viability, reduced adhesion, enhanced migration and invasiveness of the transformed cells [3]. The human placenta, although a naturally invasive structure, it is regulated by many factors such as growth factors and their binding proteins, extracellular matrix components and adhesion molecules directly at the fetal-maternal interface [22].

The etiologic factors attributed to early pregnancy loss are wide and varied including genetic, immunologic, endocrine, psychogenic, environmental, occupational, infectious and uterine causes [23]. The results showed that HPV-16 interferes with the implantation process, reducing trophoblast adhesion capabilities and altering its invasive migratory properties. This suggests that HPV may play an important role as an infectious etiology of early pregnancy loss. The mechanisms involved in the HPV-related implantation failures remain unknown. Based on this study and work from other investigators, it is postulated that HPV oncoproteins caused aberrant imprinting in the cells [24] in a similar manner observed in herpes infections [25]. It is also hypothesized that the expressed HPV-16 E5, E6 and E7 oncoproteins hypermethylate the SOX2 allele reducing its mRNA, which results in the down-regulation of factors such as E-cadherin involved in adhesion and anchorage of cells [26, 27]. Whether or not hypermethylation in the HPV-transformed embryo caused the reduced cell adhesion and subsequent embryonic wastage remains to be elucidated.

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Conflict of interest None.

References

1. Munoz N, Castellsagué X, de Gonzalez AB, Gissmann L (2006) HPV in the etiology of human cancer. Vaccine 24:1–10
2. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds) SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations), National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2009_pops09/, based on November 2011 SEER data submission, posted to the SEER web site, 2012
3. Boulenouar S, Weyn C, Van Noppen M, Moussa Ali M, Favre M, Delvenne PO, Bex F, Noël A, Englert Y, Fontaine V (2010) Effects of HPV-16 E5, E6 and E7 proteins on survival, adhesion, migration and invasion of trophoblastic cells. Carcinogenesis 31(3):473–480 [Epub 2009 Nov 16]

4. Gomez LM, Ma Y, Ho C, McGrath CM, Nelson DB, Parry S (2008) Placental infection with human papillomavirus is associated with spontaneous preterm delivery. Hum Reprod 23:709–715
5. Sarkola ME, Grénman SE, Rintala MA, Syrjnen KJ, Syrjnen SM (2008) Human papillomavirus in the placenta and umbilical cord blood. Acta Obstet Gynecol Scand 87:1181–1188
6. You H, Liu Y, Agrawal N, Prasad CK, Edwards JL, Osborne AF, Korourian S, Lowery CL, Hermonat PL (2008) Multiple human papillomavirus types replicate in 3A trophoblasts. Placenta 29:30–38
7. Weyn C, Thomas D, Jani J, Guizani M, Donner C, Van Ryssel-berge M, Hans C, Bossens M, Englert Y, Fontaine V (2011) Evidence of human papillomavirus in the placenta. J Infect Dis 203(3):341–343
8. Manavi M, Czerwenka KF, Schurz B, Knogler W, Kubista E, Reinold E (1992) Latent cervical virus infection as a possible cause of early abortion. Gynakol Geburtshilfliche Rundsch 32:84–87
9. Hermonat PL, Han L, Wendel PJ, Quirk JG, Stern S, Lowery CL, Rechtin TM (1997) Human papillomavirus is more prevalent in first trimester spontaneously aborted products of conception compared to elective specimens. Virus Genes 14:13–17
10. Uribarren-Berrueta O, Sánchez-Corona J, Montoya-Fuentes H, Trujillo-Hernández B, Vásquez C (2012) Presence of HPV DNA in placenta and cervix of pregnant Mexican women. Arch Gynecol Obstet 285(1):55–60
11. Brackmann KH, Green M, Wold WS, Rankin A, Loewenstein PM, Cartas MA, Sanders PR, Olson K, Orth G, Jablonska S, Kremsdorf D, Favre M (1983) Introduction of cloned human papillomavirus genomes into mouse cells and expression at the RNA level. Virology 129:12–24
12. Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D (2007) Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. Hum Reprod Update 13:121–141
13. Hirano T, Higuchi T, Katsuragawa H, Inoue T, Kataoka N, Park KR, Ueda M, Maeda M, Fujiwara H, Fujii S (1999) CD9 is involved in invasion of human trophoblast-like choriocarcinoma cell line, BeWo cells. Mol Hum Reprod 5(2):168–174
14. Meissner JD (1999) Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. J Gen Virol 80: 1725–1733
15. Tungteakkhun SS, Filippova M, Neidigh JW, Fodor N, Duerksen-Hughes PJ (2008) The interaction between human papillomavirus type 16 and FADD is mediated by a novel E6 binding domain. J Virol 82:9600–9614
16. Rowland SC, Jacobson JD, Patton WC, King A, Chan PJ (2003) Dual fluorescence analysis of DNA apoptosis in sperm. Am J Obstet Gynecol 188:1156–1157
17. Liu Y, You H, Chiriva-Internati M, Korourian S, Lowery CL, Carey MJ, Smith CV, Hermonat PL (2001) Display of complete life cycle of human papillomavirus type 16 in cultured placental trophoblasts. Virology 290:99–105
18. Wang J, Mayernik L, Armant DR (2007) Trophoblast adhesion of the peri-implantation mouse blastocyst is regulated by integrin signaling that targets phospholipase C. Dev Biol 302:143–153
19. You H, Liu Y, Carey MJ, Lowery CL, Hermonat PL (2002) Defective 3A trophoblast–endometrial cell adhesion and altered 3A growth and survival by human papillomavirus type 16 oncogenes. Mol Cancer Res 1:25–31
20. You H, Liu Y, Agrawal N, Prasad CK, Chiriva-Internati M, Lowery CL, Kay HH, Hermonat PL (2003) Infection, replication, and cytopathology of human papillomavirus type 31 in trophoblasts. Virology 316:281–289
21. Whiteside MA, Siegel EM, Unger ER (2008) Human papillomavirus and molecular considerations for cancer risk. Cancer 113:2981–2994
22. Chakraborty C, Gleeson LM, McKinnon T, Lala PK (2002) Regulation of human trophoblast migration and invasiveness. Can J Physiol Pharmacol 80(2):116–124
23. Rock JA, Zacur HA (1983) The clinical management of repeated early pregnancy wastage. Fertil Steril 39(2):123–140
24. Terra AP, Murta EF, Maluf PJ, Caballero OL, Brait M, Adad SJ (2007) Aberrant promoter methylation can be useful as a marker of recurrent disease in patients with cervical intraepithelial neoplasia grade III. Tumori 93:572–579
25. Long BR, Erickson AE, Chapman JM, Barbour JD, Vu BA, Ho EL, Lanier LL, Sauer MM, Carvalho KI, Nixon DF, Kallas EG (2010) Increased number and function of natural killer cells in human immunodeficiency virus 1-positive subjects co-infected with herpes simplex virus 2. Immunology 129:186–196
26. Li AS, Siu MK, Zhang H, Wong ES, Chan KY, Ngan HY, Cheung A (2008) Hypermethylation of SOX2 gene in hydatidiform mole and choriocarcinoma. Reprod Sci 15:735–744
27. Caberg JH, Hubert PM, Begon DY, Herfs MF, Roncarati PJ, Boniver JJ, Delvenne PO (2008) Silencing of E7 oncogene restores functional E-cadherin expression in human papillomavirus 16-transformed keratinocytes. Carcinogenesis 29:1441–1447