Human papilloma virus DNA exposure and embryo survival is stage-specific

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Purpose: Human papillomavirus (HPV) has been shown to disrupt late-stage implanting embryos. The objectives were (a) to assess the development of early embryos exposed to HPV DNA and (b) to analyze the blastocyst hatching process after HPV exposure.

Methods: The study involved exposing two-cell and 4–8-cell mouse embryos to DNA fragments from either HPV type 16, type 18 or DQA1 (control). The embryos were incubated for 120 h and assessed.

Results: HPV 16 and 18 inhibited two-cell embryo development. In contrast, delaying the exposure of HPV DNA until the 4–8-cell stage resulted in further embryonic development. There was 25.9% less blastocyst formed with HPV 16 exposure. Additionally, there were 25.9–31.8% more degenerated embryos with HPV 16 exposure.

Conclusions: The study demonstrated embryo stage-specific effects of HPV on early development. The results suggested HPV exposure was linked to two-cell embryo demise and delaying the exposure of HPV until later embryo stages permitted embryo development. HPV 16 was shown to decrease blastocyst formation while HPV 18 inhibited the blastocyst hatching process.

KEY WORDS: HPV; human papillomavirus; embryogenesis; preimplantation embryo.

INTRODUCTION

Human papillomavirus (HPV) is an epitheliotropic DNA virus so called because certain types cause warts or papillomas. There are more than 100 types of HPV virus (1,2). Low-risk HPV types include 6 and 11 while high risk types include 16, 18, 31, 33, 35, 39, 52, 56, and 58 (3). HPV has been linked to cervical neoplasia and carcinoma. However, the role of HPV in the etiology of spontaneous abortion or miscarriage remains contentious (4–8). HPV has been

shown to target embryonic trophoblast cells (4). The etiology of spontaneous abortion has been linked to fetal genetic abnormalities or viral infections involving cytomegalovirus (9), Epstein–Barr virus (10), herpes simplex (11), mumps (12), and rubella (13).

Little is known about HPV and early embryos. The HPV DNA can be internalized into embryos through endocytosis along with the membrane surface integrin receptor, $\alpha 6 \beta 4$ (14). In this study, the null hypothesis was that HPV DNA did not affect early embryo development. The objectives were (a) To assess the development of embryos exposed to either HPV 16 or HPV 18 E6–E7 DNA fragments and (b) to analyze the blastocyst hatching process after HPV exposure. The results would provide clinicians with a better understanding of the role HPV in spontaneous abortion. Since human embryos were not used for obvious reasons, the experimental model chosen was the mouse embryo.

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MATERIALS AND METHODS

Treatment of Mouse Embryos

Cryopreserved two-cell stage mouse embryos (20 per straw) were obtained from a commercial source (Embryotech Laboratories, Wilmington, MA) and thawed according to the supplier's instruction. Briefly, the frozen embryos in each straw were warmed, rinsed in culture medium (G1.3, Vitrolife Fertility Systems, Göteborg, Sweden) and pooled for the randomization protocol. Embryonic cell stages were visually confirmed using phase contrast microscopy.

The first group of two-cell embryos were placed into prewarmed culture medium (37°C, 1 mL) at the center of double-welled Falcon culture dish (3037, Becton Dickinson and Co., Oxnard, CA). Water was placed in the outer moat of the culture dishes to maintain humidity. The two-cell embryos were incubated at 6% CO₂ in air mixture at 37°C for 24 h to permit development to the 4–8-cell stage embryos before exposure to the HPV DNA fragments.

The second group of freshly thawed two-cell embryos were divided (6–15 embryos per treatment group) and placed into double-welled Falcon culture dishes (1 mL in each dish) with water in the outer moat. The experimental treatment consisted of pipetting 10 L of either (a) control HLA-DQA1 (designated DQV in previous nomenclature) DNA fragments, 242 bp, (b) HPV type 16 DNA fragments, or (c) HPV type 18 DNA fragments, into the two groups of embryos (two-cell or 4–8-cell) and incubated at 6% CO₂ in air mixture at 37°C for 120 h with periodic observation. The control based on the HLA-DQA1 gene belonged to the HLA-D or HLA class II polymorphic genes on chromosome 6 (15) which encoded for the V-chain of heterodimer membrane glycoproteins that display antigen peptides to the T cell receptor of CD4 + T lymphocytes (16). As part of the incubation condition, water was added into the dish moat every two days to replace evaporative loss. Generally, untreated embryos would be at the hatched blastocyst stage after 120 h of incubation.

The DNA fragments for HPV types 16 (98 bp) and 18 (80 bp) were from the E6–E7 exon region and were about the same size (17). The culture medium containing the HPV fragments was prepared by diluting 1:20 the PCR-amplified HPV products of previous PCR runs with culture medium for a final concentration of about 0.01 g DNA/L. The previous PCR runs were targeted at the HPV gene sequences for types 16 and 18 using paraffin embedded cervical

condyloma tissues as DNA templates and published primers (17–19). The PCR-amplified products were confirmed to contain HPV DNA fragments for the two HPV types using 5% polyacrylamide gel electrophoresis and ethidium bromide staining techniques prior to their usage.

After incubation, embryo morphology was assessed using an inverted microscope and specific cell stages recorded for the embryos in each treatment group. Degenerated embryos were determined by means of combined Hoechst 33342 and propidium iodide viability stains protocol (20). In addition, embryos developing to the blastocyst stage after 120 h of incubation were considered viable. The blastocysts were grouped into early, expanded, hatching or hatched stages. Hatching blastocysts were characterized by the extrusion of pluripotent cells out of the single fenestra in the thinned zona pellucida. Features of hatched blastocysts included the explanted embryo mass on the dish and the empty zona pellucida shell. The hatching and hatched blastocysts were grouped together as hatched blastocysts and represented advanced development.

Statistical Analysis

The results were presented as percentages of development to the blastocyst, hatching blastocyst or degenerated stages. Significant difference was tested using chi-square statistics Epistat program (Epistat Services, Richardson, TX). A difference with $p < 0.05$ was considered significant.

RESULTS

Development of the two-cell embryos to the blastocyst stage was completely inhibited by both HPV 16 and 18 exogenous E6–E7 DNA fragments (Table I). In contrast, delaying the exposure of HPV 18 DNA to the embryos until the 4–8-cell stage resulted in further embryonic development to the blastocyst stage. However, exogenous DNA from HPV type 16 partially inhibited the 4–8-cell embryo development to blastocysts. Embryos incubated in the presence of the DQA1 (control) DNA fragments continued development to the blastocyst stage. In terms of degeneration, exposure of two-cell embryos to HPV 16 DNA resulted in more degenerated embryos (31.8%) when compared with embryos exposed to HPV 18 DNA. A similar result was obtained in the 4–8-cell embryo group.

Table I. Stage-Specific Development of 2-Cell or 4–8-Cell Mouse Embryos Exposed to Exogenous DNA Derived From the E6–E7 Region of Papillomavirus (HPV) Types 16 and 18

Treatment	Development to blastocyst (%)	Continued to hatching (%)	Number of embryos degenerated (%)
Exposure at 2-cells stage			
Control DQA1	19/20 (95.0)	12/19 (63.2)	1/20 (5.0)
HPV 16	0/21 (0)*	0/0 (0)	21/21 (100.0)
HPV 18	0/22 (0)*	0/0 (0)	15/22 (68.2)
Exposure at 4–8-cells stage			
Control DQA1	20/22 (90.9)	10/20 (50.0)	2/22 (9.1)
HPV 16	14/28 (50.0)*	7/14 (50.0)	14/28 (50.0)*
HPV 18	22/29 (75.9)	5/22 (22.7)*	7/29 (24.1)

* $p < 0.05$. different from control.

There were no hatching blastocysts in the HPV-exposed two-cell embryo groups. However, delaying the exposure of HPV DNA to the embryos until the 4–8-cell stage resulted in similar percentages of hatching blastocysts in the HPV 16 DNA and control embryos. Interestingly, there were significantly less hatching blastocysts (28.3%) in the HPV 18 DNA-exposed 4–8-cell embryo group when compared with the control group.

DISCUSSION

The present study demonstrated an embryo stage specific response to the presence of E6–E7 derived HPV DNA. Furthermore, the response was specific to the type of HPV. Differential effects of the various HPV types on cells have been documented (21). Both, types 16 and 18 tested here were deleterious to the two-cell embryo and caused embryo demise. The results suggested an association between the presence of E6–E7-derived HPV DNA and early embryonic loss. The HPV E6–E7 are early genes associated with cell transformation (3). The expressed oncoprotein of HPV E6 degrades p53 protein through the cellular ubiquitin-protein ligase E6-AP pathway (22, 23). The E7 oncoprotein binds to the retinoblastoma gene products, pRb, p107, cyclin A, AP-1 transcription factor, and the TATA box binding protein, TBP (24–26). The overall result is cellular instability.

There is very little information on the pathogenesis of HPV in early preimplantation embryos. Unlike multicellular organisms, the early two-cell embryo either had only two copies of the genome that became disrupted by the HPV DNA or it lacked the necessary repair mechanism to correct for the integrated HPV DNA. The sites for HPV DNA integration into the host genome have been reported to be near oncogenes (27) causing transformation of cells, or at the chromosomal fragile site (28–32)

at 3p14 (31,33) and the FRA8C site at 8q24 (34) resulting in gene interruption and loss of chromosome heterozygosity (24,35). Indirect evidence linking HPV infection and embryo demise would be the prevalence of HPV infection in the infertile population. This has been reported to be from 4–5.5% (36) to 7.8% (37) and up to 100% (38) of the infertile population (39). However, documenting HPV infection in the infertile population is tenuous due to the latent nature of embryonic loss.

In this study, delaying the exposure of the viral DNA fragments until the 4–8-cell embryo stage permitted the embryos to overcome developmental inhibition associated with the exogenous HPV DNA. In particular, 4–8-cell embryos exposed to HPV 18 DNA had comparable blastocyst development similar to the control, albeit the hatching process was significantly reduced. Many embryos exposed to HPV 16 DNA did not reach the blastocyst stage. The embryos became apoptotic and shrank suggesting that the presence of HPV 16 resulted in greater embryonic loss or infertility. Interestingly, a survey of the infertile population indicated a greater prevalence of HPV 16 in the infertile population although the authors did not associate HPV with infertility (40).

HPV-16 exposed embryos that reached the blastocyst stage continued to develop and hatched unlike HPV 18-exposed embryos. This suggested that HPV 18 blocked genes needed for the blastocyst hatching process implying a self-elimination process. Previous studies in blastocysts have suggested that the HPV DNA taken into blastocyst cells resulted in host DNA fragmentation (21). An analysis of HPV-affected blastocysts in utero would not be possible for obvious reasons. However, Hermonat and colleagues (5) showed that first trimester spontaneously aborted fetal tissues were infected with HPV (60% versus 20% in the control elective specimens). Previous reports indicated that 31–70% of spontaneous

aborted cases were positive for either HPV 16 or 18 (5–7). The HPV DNA were detected mainly in the syncytiotrophoblasts and sporadically in the cytotrophoblasts and stromal cells (4).

In summary, the study showed embryo stage specific effects of HPV DNA on early development. The results suggested an association between HPV exposure and two-cell embryo demise. Furthermore, delaying the exposure of HPV until later embryo stages abrogated some of the deleterious effect of HPV on embryos. HPV 16 was shown to decrease blastocyst formation while HPV 18 inhibited the blastocyst hatching process. The present study focused on HPV 16 and 18 and the possible involvement of other HPV types in disrupting embryo development was not ruled out. The study suggests the need for increased vigilance to detect HPV infection during early pregnancy, particularly when accompanied by a history of embryonic loss and HPV vaccine considerations as part of the infertility treatment.

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