Human Papillomavirus and Blastocyst Apoptosis¹

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Purpose: The effect of human papillomavirus (HPV) DNA from the E6-E7 region on the integrity of DNA in blastocyst stage embryonic cells was studied. The study design paralleled the event whereby HPV DNA from the infecting virus would target host cell DNA. The objectives were (a) to determine if the DNA of blastocysts were disrupted by the presence of HPV DNA and (b) to determine if the intensity of DNA damage was associated with the type of HPV.

Methods: This study involved superovulating female mice, mating, collecting one-cell embryos, and culturing to the expanded blastocyst stage. The blastocysts were infected with PCR-synthesized DNA fragments from either HPV type 16, 18, 31, or 33. The blastocyst DNA were analyzed by comet assay after 24 h of incubation. The fluorescent images were digitized and the pixel intensity of each blastocyst was measured.

Results: Only the DNA of HPV type 16 was associated with significant DNA fragmentation in comparison with the other HPV types. There was no relationship between HPV DNA fragment size and the intensity of DNA fragmentation.

Conclusions: The data suggested that one mode of action of HPV type 16 was to initiate apoptosis of embryonic cells through DNA fragmentation. The effect of HPV 16 occurred rapidly within 24 h. The intensity of DNA damage was not linked to the specific type of HPV. However, the results do not rule out the other HPV types affecting embryos under conditions different from this study.

KEY WORDS: Blastocyst; comet assay; embryo; human papillomavirus; HPV; single cell gel electrophoresis.

INTRODUCTION

Recently, embryonic trophoblast cells were shown to be the predominant target of human papillomavirus (HPV) type 16 (1). The authors suggested that HPV-infected trophoblasts led to spontaneous abortion. Present understanding of the etiology of

spontaneous abortion or embryonic wastage includes genetic abnormalities or viral infections involving cytomegalovirus (2), Epstein-Barr virus (3), herpes simplex (4), mumps (5), and rubella (6). The role of HPV in causing spontaneous abortion remains controversial. (1,7–10). One possible mechanism may involve a disruption of the embryonic DNA genome by HPV, but information on this mechanism is lacking.

Several studies have suggested that embryos at the blastocyst stage possess the capacity to take up exogenous HPV DNA molecules (11–12). The HPV DNA is internalized through endocytosis along with the membrane surface integrin receptor, $\alpha 6 \beta 4$ (13). The hypothesis in this study was that HPV DNA do not affect embryo genome. The objectives were (a) to determine if the DNA of embryos at the blastocyst stage were disrupted by the presence of HPV DNA

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derived from the E6-E7 region and (b) to determine if the intensity of DNA damage in the blastocysts was related to the type of HPV. The results would provide clinicians with a better understanding of the role of HPV in spontaneous abortion. Since human embryos cannot be used for obvious reasons, the experimental model chosen was the mouse blastocyst. The amount of DNA damage was determined using the modified comet assay or single cell gel electrophoresis assay.

MATERIALS AND METHODS

Collection of Mouse Blastocysts

Female mice were hormonally stimulated with 5 IU pregnant mares serum gonadotropin (PMSG; Sigma, St. Louis, MO) followed by 5 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, MO) and mating with a proven male 2 days later (14). The protocol described here was in compliance with animal use guidelines and was approved by the Institutional Animal Care and Use Committee. Pronuclear stage embryos were flushed from the fallopian tubes 20 h after mating. The one-cell fertilized oocytes (zygotes) were incubated (15) in bicarbonate-based synthetic human tubal fluid medium (HTF, Irvine, Santa Ana, CA) containing 10% synthetic serum substitute (SSS, Irvine, Santa Ana, CA) until they reached the hatching blastocyst stage after about 100 h of incubation. Embryonic stages were visually confirmed using phase contrast microscopy. The experiment was conducted in duplicate by superovulating a second batch of mice, collecting and growing embryos, HPV exposure, and image analyses.

Each group consisted of 5–10 randomly-assigned blastocysts taken from a mixed pool of embryos derived from five different female mice used on each of the two occasions. The blastocysts were incubated in the presence of either HPV type 16, 18, 31, or 33 DNA fragments for 24 h at 37°C in 5% CO₂ in air mixture. Another group of blastocysts was incubated without the HPV DNA fragments (control). The DNA fragments for HPV types 16 (98 bp), 18 (80 bp), 31 (162 bp), and 33 (103 bp) were about the same size (16). The supplemented HTF medium (15) with the HPV fragments was prepared by diluting the PCR-amplified HPV products of previous PCR runs with HTF medium (1:20) for a final concentration of about 0.01 µg DNA/µL. The previous PCR runs were targeted at HPV gene sequences for

types 16, 18, 31, and 33 by using paraffin embedded cervical condyloma tissues as DNA templates and published primers (16–18). The PCR-amplified products were confirmed to contain HPV DNA fragments for the four different HPV types using 5% polyacrylamide gel electrophoresis and ethidium bromide staining techniques prior to their usage in the blastocyst incubation step. At the end of the incubation period, each group of blastocysts was separately washed and pipetted on to a clean glass slide and air-dried.

Modification of the Comet Assay for Fixed Blastocyst Cells

The entire procedure was carried out with the room lights off or in diffused lighting. The air-dried blastocysts on the glass slides were first fixed in methanol solution (Fixative Solution, Diff-Quik, Dade Behring, Newark, DE) for 15 s and air-dried. The fixed blastocysts were then stained for 5 min in freshly prepared acridine orange solution. This solution was prepared by dissolving 0.2 mg acridine orange (Catalog No. 10390, United States Biochemical Corp., Cleveland, OH) in 1.0 mL purified water.

The slides were washed with water to remove background staining and air-dried. Self-adhesive sticker labels (Catalog No. 43-640, Presaply, Dennison, Framingham, MA) were cut into strips and pasted to form a rectangular frame around the stained cells on each glass slide. The stickers served to anchor the mini-agarose gel layer. The 0.8% agarose was prepared by mixing 0.8 g agarose (Catalog No. 15510-019, Life Technologies, GIBCO BRL, Grand Island, NY) in 100 mL of the 1× TBE solution (13.5 g Tris-HCl pH 7.5, 6.8 g Boric Acid, 5 mL of 0.5 M EDTA in 1250 mL purified water), heating until dissolved, and cooling to 45°C. The agarose was pipetted on to each glass slide surface placed at a 20° angle and excess agarose allowed to run off the edges. Thinner agarose layers tended to be easier to work with under the microscope high powered objective. A cover slip was not used. Each slide was placed horizontal on the table. Once the agarose had solidified, the slides were placed in 4°C alkaline lysis buffer (1% N Lauroylsarcosine, 1.0 M Tris-HCl pH 7.5, 0.5 M EDTA, 0.3 M mercaptoethanol, pH adjusted to >10 with sodium hydroxide pellets) for 1 h to release and unwind cell DNA. Then, the slides were rinsed with purified water and placed submerged (1–2 mm from the surface) in 1× TBE solution in the electrophoresis chamber

Fig. 1. Modified alkaline comet assay of dried mouse blastocyst. Each fixed blastocyst was stained in acridine orange, layered over with a mini-agarose gel, lysed, processed through electrophoresis, and viewed under fluorescent microscopy. The images were captured using an inexpensive videocam computer camera held to the microscope eyepiece and converted to gray-scale for density analysis using an image program. The images showed a mixture of trophoblast and inner cell mass DNA material since only nuclei acids were stained. The control blastocyst (A) was darker in overall density compared with the HPV type 16 treated blastocyst. (B) Lighter DNA density represented increased fragmentation of the DNA.

for 5–10 min to equilibrate. Following this, constant voltage at 50 V was applied for 40 min.

Excess solution was blotted from each slide and the slide was immediately examined using an ultraviolet (UV) fluorescent microscope at 500 $\times$ magnification. The images were captured by placing an inexpensive QuickCam Pro camera (Logitech, Fremont, CA) over the microscope eyepiece and saving the 640 $\times$ 480 pixels images to hard-disk. The color of the images and background were inverted using the Windows 98 Microsoft Paint program, the entire image saved in grey-scale (Fig. 1), and the average pixel intensity for each entire blastocyst analyzed using Paint Shop Pro 6 software (Jasc Software, Eden Prairie, MN). A 30-day evaluation copy of Paint Shop Pro may be downloaded at shareware websites. For accuracy, each cell image was corrected for differences in background intensity.

Statistical Analysis

The results were presented as mean pixel intensity ± 1 standard deviation. Data on the mean intensity of all the nuclei for each blastocyst regardless of type in the various HPV groups were compared and tested using the Student's *t*-test statistic. A difference with $P < 0.05$ was considered significant.

Table I. Modified Comet Assay Detection of Differences in DNA Fragmentation in Mouse Blastocysts After 24 h In Vitro Exposure to Exogenous DNA Derived from the E6-E7 Region of Papillomavirus (HPV) Types 16, 18, 31, and 33

Category	DNA fragment size (bp)	N	DNA fragmentation ^a (mean pixel intensity $\pm$ 1 SD)
Control	N/A	12	190.1 $\pm$ 24.4
HPV type 16	98	6	165.7 $\pm$ 10.1*
HPV type 18	80	9	180.1 $\pm$ 21.7
HPV type 31	162	8	171.9 $\pm$ 33.2
HPV type 33	103	10	179.5 $\pm$ 12.0

^aLower pixel intensity is associated with more DNA fragmentation.

* $P < 0.05$; different from control.

RESULTS

There was significantly higher fragmentation of mouse blastocyst DNA after exposure to DNA fragments from HPV type 16 in comparison with the control and also with the other 3 HPV types (Table I). HPV type 16 has been linked to the etiology of malignant cervical cancer cases. Another HPV type associated with malignancy, HPV 18, did not affect blastocyst DNA. The remaining HPV types associated with moderate forms of cervical cancer, HPV 31 and 33, also did not affect blastocyst DNA. There was no relationship between HPV DNA fragment size and the amount of DNA fragmentation in the nuclei of blastocyst cells.

DISCUSSION

The DNA of mouse blastocysts was significantly fragmented or damaged in the presence of specific viral DNA. In this case, DNA from the E6-E7 region of HPV type 16 started apoptosis of the blastocyst within 24 h of exposure. This result was consistent with the observation of a three-fold higher prevalence of HPV 16 in spontaneous aborted tissue material when compared with elective aborted tissue material (1,7). Interestingly, under light microscopy, the morphology or appearance of the HPV-treated blastocysts were not different from the control blastocysts.

In terms of host DNA fragmentation, the HPV DNA of types 18, 31, and 33 were without effect on the DNA of the blastocyst cells. This suggested that either the DNA from these HPV types, by mode of action, did not disrupt the blastocyst cell DNA or that the HPV DNA fragments were not taken up into the cells during endocytosis (13). Although differences in

the uptake of exogenous DNA by blastocysts have been reported (11), for example reduced uptake in the case of HPV 18 DNA, it did not explain the lack of an effect by HPV types 31 and 33 that were both readily taken into the blastocyst (18).

The in vitro data showing the fragmentation of blastocyst DNA by exogenous HPV DNA type 16 suggested that this HPV might be one of the factors involved in embryonic wastage or early spontaneous abortion. The clinical significance of this finding is the need for increased vigilance to detect HPV infection during early pregnancy or during treatment for infertility, particularly when recurrent miscarriages are involved. It should be noted that the present study was based on mouse and not human blastocyst for obvious reasons. In addition, in vivo studies based on the products of conception at the implanting blastocyst stage are not accessible for analysis for the presence of HPV. However, Hermonat and colleagues (6) analyzing fetal material from later stages such as spontaneous abortions in the first trimester showed that a high percentage of cases were infected with this virus (60% vs. 20% in the control elective specimens). Previously, there had been reports (7) that 31–70% of spontaneous aborted cases were positive for either HPV 16 or 18 (9). The HPV DNA were detected mainly in the syncytiotrophoblasts and occasionally in the cytotrophoblasts and stromal cells (1).

Studies using various cell lines have shown that the DNA of HPV 16 or 18 integrate near oncogenes (19) causing transformation of cells, or integrate into fragile sites located on the chromosomes (20–24) causing gene interruption and loss of chromosome heterozygosity (25–26). Another consequence of HPV DNA uptake into the host cell would be the fragmentation of the host DNA or apoptosis as demonstrated here by the effect of HPV 16 on blastocyst DNA. However, an association between increased prevalence of HPV and miscarriage rates over the past 40 years could not be made because the techniques to detect HPV differed over the years.

The novel modification of the comet assay or single cell gel electrophoresis for the analysis of blastocyst cells was presented in this study. The comet assay was originally introduced in 1984 by Östling and Johanson (27) for the analysis of DNA fragmentation in individual white blood cells. The broken pieces of DNA loops unwinding from the relaxed supercoiled nucleus gave the appearance of a comet tail, hence the name “comet assay.” The authors used neutral electrophoresis buffer conditions to show that the migration of double-stranded DNA loops from the

damaged cell nucleus was proportional to the extent of the damage inflicted on the cell. The comet assay was modified by Singh and colleagues in 1988 (28) by using alkaline electrophoresis buffers to expose alkali-labile sites that increased assay sensitivity (29). The comet assay has been modified several times since its introduction (30–32).

The original comet assay was based on initially living cells suspended in a mini-agarose gel (27). In this laboratory, the assay was adapted for dried, fixed blastocysts attached on glass slides and overlaid with mini-agarose gel layers. The comet assay usually involved the analysis of the comet tail, tail moment, or more recently the intensity of the nuclear DNA such as the sperm head (33). In the present study, the overall intensity of the DNA in each whole blastocyst was measured. There were no attempts to separate the individual cells of the blastocysts such as cells of the trophoblast or inner cell mass. The reason for this was because the isolation of individual cells required immunochemical manipulation that would yield artifacts. However, a disadvantage of whole blastocyst analysis, in contrast to individual cells, was that the cell type affected most by the viral DNA could not be ascertained. Nevertheless, the overall DNA intensity of each whole blastocyst was proportional to the DNA integrity and a reduced intensity would suggest the presence of fragmentation.

In summary, the comet assay was modified to analyze DNA integrity in blastocyst cells after in vitro exposure to DNA fragments from HPV types 16, 18, 31, and 33. The in vitro data showed DNA fragmentation in blastocysts after 24-h exposure to HPV DNA type 16 suggesting that the mode of action of HPV type 16 involved initiating apoptosis through disruption of DNA in the embryo. Although HPV types 18, 31, or 33 had no effect on blastocyst DNA integrity under the conditions of the study, the involvement of these HPV types in disrupting embryo development through other mechanisms was not ruled out.

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