Transfection of the Inner Cell Mass and Lack of a Unique DNA Sequence Affecting the Uptake of Exogenous DNA by Sperm as Shown by Dideoxy Sequencing Analogues

MILAGROS CABRERA,[1] PHILIP J. CHAN,[1–3] THERESA H. KALUGDAN,[1] and ALAN KING[1]

Submitted: August 22, 1996
Accepted: September 30, 1996

Purpose: *The purpose of this study was to determine whether exogenous DNA internalized into blastocysts after transference from DNA-carrier sperm are localized at the inner cell mass or trophoblast cells and to identify differences in uptake of exogenous DNA fragments by sperm due to unique DNA sequences.*

Methods: *Mouse blastocysts at the hatching stage were exposed to migrating human sperm cells carrying exogenous DNA fragments synthesized from the E6–E7 conserved gene regions of human papillomavirus (HPV) types 16 and 18. After an interaction period of 2 hr, the transfected blastocysts were washed several times to remove extraneous sperm and the blastocysts were dissected into groups of cells derived from the inner cell mass and trophoblasts. The cells were analyzed by polymerase chain reaction (PCR) for the presence of HPV DNA fragments. In the second part of the experiment, thawed donor (N = 10) sperm cells were pooled, washed, and divided into two fractions. The first (control) fraction was added with formalin and further divided and added with a ^{35}S-radiolabeled G, A, T, or C sequencing mixture. The second fraction was similarly treated but the formalin step was omitted from the treatment. After an hour of incubation at 37°C, the sperm specimens were washed several times by centrifugation and DNA extracted by the GeneReleaser method. The extracted DNA were processed on sequence gels, and the autoradiographs analyzed.*

Results: *Mouse blastocysts transfected by carrier sperm with DNA from HPV types 16 and 18 showed localization of the HPV DNA to both the inner cell mass and trophoblast cells. Negative controls consisting of untreated human sperm and untreated mouse blastocysts did not reveal any evidence of HPV DNA. The positive sperm control generated expected DNA fragments from HPV types 16 and 18. In the second experiment, the intensities of the DNA fragments in the G, A, T, and C columns from low to high molecular weights were not different from the positive control bands. Band intensities of the four sequencing columns were similar. Formalin pretreatment of the sperm inhibited uptake of the DNA fragments from the smallest to the largest DNA molecules.*

Conclusions: *Exogenous DNA taken into blastocysts are localized to both the inner cell mass and trophoblast cells. Only live sperm exhibited the capacity to carry various sizes of exogenous DNA, suggesting the involvement of active cell membrane mechanism in the transference process. The results showed that DNA fragments terminating in any of the four nucleotides were equally taken up by the sperm cell. Fragments of DNA produced by the sequencing reaction failed to identify a unique DNA sequence that would facilitate or inhibit the sperm from taking up exogenous DNA.*

KEY WORDS: spermatozoa; inner cell mass; blastocysts; human papillomavirus; sequencing reactions.

INTRODUCTION

It has been demonstrated that sperm cells can serve as vectors for exogenous DNA fragments (1–6). The targets for these DNA-carrier sperm include oocytes (1–3) and embryos (6). Using radiolabeling methods, the absorbed exogenous DNA fragments were localized to the postacrosomal and equatorial regions of the sperm head (5). Some of the DNA fragments are internalized into the compact sperm head (5), while the remaining excess DNA fragments are localized externally at the membrane surface, making it possible to transfect the plasma membrane of embryonic cells at the time of contact (6). This represents a potential problem in vivo for the inadvertent transfer of exogenous DNA originating from viruses, bacteria, or other genomic sources from the migrating sperm to the preimplantation embryo in the reproductive tract. Cur-

[1] Department of Gynecology and Obstetrics, Loma Linda University School of Medicine, Loma Linda, California 92350.
[2] Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, California 92350.
[3] To whom correspondence should be addressed at Department of Gynecology and Obstetrics, Loma Linda University School of Medicine, Loma Linda, California 92350.

1058-0468/97/0200-0120$12.50/0

rently, there is considerable interest in determining what DNA sequences facilitate or inhibit DNA absorption and whether the absorbed DNA is taken up into the trophoblast cells or the cells of the inner cell mass (ICM), as these two cell groups ultimately give rise to the placental and fetal components, respectively. Hence, the objectives were (a) to determine whether exogenous DNA internalized into blastocysts are localized at the inner cell mass and/or the trophoblast cells and (b) to identify differences in uptake of exogenous DNA fragments by sperm due to unique DNA sequences or molecular size of the fragments. To accomplish the second part of the objectives, a mixture of random radiolabeled fragments generated through sequencing reactions was added to the sperm cells. The idea was that in analyzing the DNA fragments taken up by the sperm in a sequence gel, any interruptions or enhancements in the sequence will suggest a unique terminating code affecting DNA uptake. The data obtained will provide further information on sperm-mediated pathogenesis of reproductive and embryonic tissues as well as the possibility of producing transgenic progeny through artificial insemination.

MATERIALS AND METHODS

Sperm Transfection of Blastocysts

The procedures for transfecting blastocysts either by incubation in the presence of DNA fragments in the culture medium or by means of carrier sperm have been reported previously (6,7). In this study, the latter procedure using carrier sperm was used. Briefly, donor semen (6) testing negative for human papillomavirus (HPV) was mixed with an equal volume of protein-free HEPES-modified HTF (Irvine Scientific, Irvine, CA) culture medium and was centrifuged for 30 sec at 9600 *g* at room temperature. The resultant sperm pellet was resuspended in 1.0 ml culture medium and verified to have a sperm motility of >50% and a count of >20 million/ml.

A solution containing the mixture of two types of DNA fragments (1 μg/μl each) from HPV types 16 and 18 was added into the single tube containing the washed sperm cells (1:1 volume mixture). The HPV DNA fragments were previously synthesized from the polymerase chain reaction (PCR) using primers specific for these HPV types as reported previously: type 16, 98 base pair (bp); and type 18, 80 bp (6,8). All HPV primers target the E6–E7 open reading frame (ORFs) region, which has been shown to remain intact during integration into the genome (9).

The sperm cells were incubated for 1 hr at 37°C in an air incubator to allow the sperm to take up the HPV DNA fragments, followed by centrifuge washing (three times at 9600 *g*, 30 sec) to remove any extraneous DNA fragments, and an aliquot (10 μl) of the HPV-coated sperm was injected into the culture medium droplet immersed under nontoxic mineral oil in a large petri dish. The culture medium droplets (500 μl each) in this petri dish were HEPES-modified HTF containing 10% human fetal cord serum (Lot No. 1338, Gemini Bioproducts, Inc., Calabasas, CA). Using an arrangement that was previously proven effective (6), narrow-bore (0.1-mm diameter, 2-cm length) glass tubing was used to connect a culture medium droplet to a second droplet located about 2 cm away under oil in the same petri dish. The glass tubing was prefilled with the same culture medium. The procedure for the interaction of sperm and blastocysts have been reported (6). Briefly, mouse (strain CB6F1) blastocysts at the hatching stage, developed in vitro, were pipetted into the second droplet of culture medium under oil. The transfected sperm in the first droplet were allowed to migrate through the tube and to contact the mouse blastocysts at the other end of the tube over a period of 2 hr. Migration of the sperm to the blastocysts was visually confirmed using a Nikon Diaphot inverted microscope (magnification, ×400).

The transfected blastocysts were pipetted out into a separate culture medium droplet containing 300 μl DNase I (Sigma Chemical Co., St. Louis, MO) and incubated for 30 min at room temperature (25°C) to destroy external DNA fragments. The mouse blastocysts were then rigorously washed several times to remove extraneous sperm. Each blastocyst was gently held in place at the bottom of the culture dish by pressing the tip of a 25-gauge needle attached to a 1-ml tuberculin syringe at a site precisely opposite the inner cell mass. This was readily accomplished with the help of a high-magnification dissecting microscope. Blastocysts with a small or no inner cell mass that were easily identifiable were not used. A sharp glass micropipette was used carefully to section off each inner cell mass, which was then pipetted into a separate reaction tube. The remaining trophoblast cells were pipetted into another reaction tube. The cells were analyzed by polymerase chain reaction (PCR) for the presence of HPV DNA fragments described below. In the second part of the experiment, thawed donor ($N = 10$) sperm cells were pooled, washed, and divided into two portions. The first (control) portion

of sperm was added with 10% formalin and further divided and added with a ^{35}S-radiolabeled G, A, T, or C sequencing mixture prepared from reactions with Sequenase 2, a genetic variant of bacteriophage T7 DNA polymerase (United States Biochemical, Cleveland, OH) using the vector M13mp8 (0.2 μg/μl) as control template described below. A second portion of sperm was similarly treated but omitting the formalin step. After an hour of incubation at 37°C in an air incubator, the sperm specimens were washed four times by centrifugation (9600 *g*, 30 sec), and DNA was extracted by the GeneReleaser method described below. The extracted DNA was loaded into a 6% sequencing gel in a Kodak IBI base runner apparatus (International Biotechnologies, Inc., New Haven, CT), and electrophoresis performed as described below. The experiment using sequencing gels to analyze sperm DNA uptake was repeated and the results compared and verified.

Extraction of Sperm and Blastocyst DNA

At the time of PCR analyses, the cells in their respective tubes were each added with 20 μl of GeneReleaser (Bioventures, Murfreesboro, TN), overlaid with 50 μl of mineral oil and placed in the thermocycler (Perkin Elmer Cetus, Norwalk, CT). The DNA extraction cycles were carried out as suggested by the manufacturer: 65°C for 30 sec, 8°C for 30 sec, 65°C for 90 sec, 97°C for 180 sec, 8°C for 60 sec, 65°C for 180 sec, 97°C for 60 sec, 65°C for 60 sec, and held at 80°C for 1 min. Following this preparation, PCR amplification cycles were carried out as described below.

PCR

Each reaction tube was heated for 7 min at 95°C to denature any DNA present, and its content added with PCR cocktail solution (Perkin Elmer Cetus) containing 10 m*M* Tris–HCl at pH 8.3, 50 m*M* KCl, 4.0 m*M* $MgCl_2$, 200 μ*M* of each dNTP, 100 μg/ml gelatin, a 1 μ*M* concentration of each pair of primers for HPV type 16 or 18, 2.5 U Taq polymerase enzyme (AmpliTaq, Perkin Elmer Cetus), and 50 μl mineral oil. No additional mineral oil was added for each tube of blastocysts extract. The sequences of the HPV primers have been reported previously (8). The PCR amplified a 98-bp product for type 16 and an 80-bp product for type 18.

The negative controls consisted of the complete PCR reaction cocktail reagents and untreated human sperm or untreated mouse blastocysts. The positive control consisted of the HPV DNA-carrier sperm used to infect the blastocysts. The PCR was carried out in a Perkin Elmer Cetus Thermal Cycler housed separately in a dedicated room. The cycling parameters were set for 40 step-cycles with 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C, followed by a final extension at 72°C for 7 min (7). The amplified product was extracted from each reaction tube using 100 μl of chloroform and visualized in UV light after standard 5% acrylamide gel electrophoresis and ethidium bromide staining. For each acrylamide run, the *Hae*III digest of ϕX174 served as the marker.

Sequencing Reaction and Analyses

The sequencing reaction to generate radiolabeled DNA fragments of different molecular sizes terminating in either G, A, T, or C nucleotide was based on the Sequenase 2 protocol (United States Biochemical). Briefly, the control single-stranded DNA, M13mp8, was mixed with one primer and buffer and heated at 65°C for 2 min. While the mixture was cooling, the labeling reaction was carried out with dGTP and aliquots (2.5 μl each) were divided into tubes labeled G, A, T, and C. The Sequenase 2 enzyme was diluted and added to the labeling mixture, the cooled DNA template, 0.1 *M* dithiothreitol (DTT), and radiolabeled dATP. The reaction was allowed to occur for 3 min at 0°C, followed by centrifugation, and 3.5 μl was taken out and added to each of the tubes labeled G, A, T, or C. The mixture was centrifuged and incubated for 3 min at 37°C, and 4 μl of stop solution added to each of the four tubes. The tubes were heated for 3 min at 96°C, and 4 μl of mixture removed from each tube and loaded into the 6% sequencing gel and processed for 4 hr at 2100 V. The gel was removed, fixed in a 10% methanol:10% acetic acid mixture for 15 min, and dried for 1 hr. The dried gel was placed with the Kodak X-Omat X-ray film and kept for 3 days before film processing. The intensities of the bands from low to high DNA molecular size and from DNA fragments terminating in G, A, T, or C were compared and the results recorded.

RESULTS

The results (Fig. 1) showed that exogenous DNA fragments derived from HPV types 16 and 18, delivered to mouse blastocysts by carrier sperm, were detected in both the inner cell mass and trophoblast

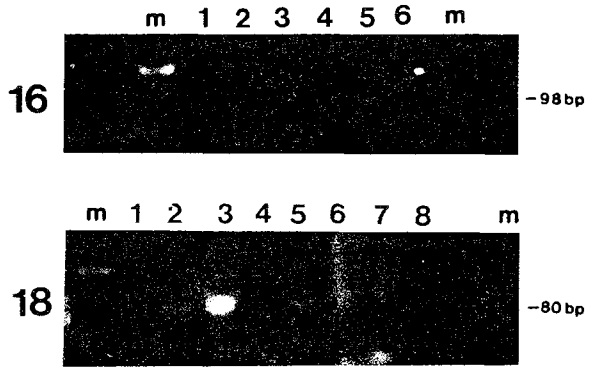

Fig. 1. Polyacrylamide 5% gel electrophoresis and ethidium bromide staining of polymerase chain reaction-amplified products targeting for human papillomavirus (HPV) types 16 (98 bp) and 18 (80 bp). HPV-16 (top): lane 1, control untreated blastocysts; lane 2, transfected whole blastocysts; lane 3, positive control consisting of the mixture of HPV DNA fragments; lane 4, negative (water) control; lane 5, trophoblast cells; lane 6, inner cell mass. HPV-18 (bottom): lane 1, negative (water) control; lane 2, inner cell mass; lane 3, trophoblast cells; lane 4, negative control untreated blastocysts; lane 5, positive control of transfected whole blastocysts; lane 6, positive control of the mixture of HPV DNA fragments. To authenticate DNase I activity, DNase I was added to the control HPV mixture in the presence of a DNase inhibitor, EDTA (lane 7), or in the absence of EDTA (lane 8). Lanes m are ϕX-174 *Hae*-III restricted DNA marker lanes.

cells. DNase I treatment of HPV DNA fragments destroyed all DNA outside the cells (no bands seen), suggesting that the transfected DNA from HPV types 16 and 18 had been internalized into the cells. Negative controls consisting of untreated humansperm and untreated mouse blastocysts did not reveal any evidence of HPV DNA. The positive sperm control generated expected DNA fragments from HPV types 16 and 18.

In the second experiment, the intensities of the DNA fragments in the G, A, T, and C columns from low to high molecular weights were not different from those in the positive control bands. The intensities of the bands in the four sequencing columns representing each of the four nucleotides were similar. Formalin pretreatment of the sperm inhibited uptake of the DNA fragments by the sperm cells regardless of the size of the DNA molecules.

DISCUSSION

The present study demonstrated the presence of exogenous DNA in cells of the ICM and trophoblast regions, suggesting that exogenous DNA transferred from the sperm to the blastocysts were rapidly distributed into these two cell types. The PCR products amplified from DNA taken from the ICM region were generally less than products amplified from the trophoblast region as shown by the less intense bands in the gel. This is due to the lower number of ICM cells in contrast to trophoblast cells. While the time needed for DNA uptake in blastocysts has not been reported, in the sperm cell it has been shown that DNA uptake occurs within the first 10 min (2,5,11). The DNase I treatment consisting of washing the transfected blastocysts did not obliterate the HPV DNA, clearly suggesting that the exogenous DNA had been internalized into the cells. The fate of the exogenous DNA taken into the cells, whether it is integrated into the host or mitochondrial genome, remains as an episome or is destroyed by endonuclease remains to be elucidated. However, studies in other cell types indicate that the genetic region in the E6–E7 open reading frames (ORFs) of HPV, the same used in this study, is readily integrated into the host genome (9). The potential for the inadvertent transfer of viral or bacterial DNA into the embryos from sperm cells needs to be considered in light of the results in this study. Conversely, it may be possible to use the DNA-carrying properties of the sperm to deliver desirable DNA to correct defective DNA in target embryos, knowing now that the ICM cells, which give rise to the fetus, will be affected in the transference process. The procedure involved may be as simple as artificial insemination during early pregnancy.

Several reports have shown that sperm cells are capable of carrying exogenous DNA (1–6) and transferring the DNA into oocytes (1–3) and embryos (6). While most investigators have focused on the capacity of sperm to serve as a vector for exogenous DNA, studies to determine the preferred molecular size of DNA fragments for transference and the specific gene sequences that facilitate or retard the uptake of DNA into the cells are lacking. In this study, using radiolabeled sequencing fragments, it was found that sperm did not exhibit a preference for absorbing DNA fragments of particular molecular sizes, suggesting that a specific membrane-bound receptor was not involved. Furthermore, neither the terminal nucleotide of the DNA fragment nor specific DNA gene sequences influenced the uptake process, suggesting that the tertiary DNA structure did not serve as a natural barrier to absorption of exogenous DNA by sperm. However, the sperm had to be alive to take up the exogenous DNA fragments as shown by the lack of DNA uptake in formalin-treated sperm confirming similar findings

in a report by Lavitrano and colleagues (2). Further studies are needed to characterize the DNA-carrying properties of the sperm before the sperm can be used as a noninvasive gene delivery system.

ACKNOWLEDGMENTS

The authors thank the staff at the Loma Linda University Center for Fertility and In Vitro Fertilization and the Loma Linda University Gynecology and Obstetrics Medical Group, Inc., for their help in the preparation of the manuscript.

REFERENCES

1. Brackett BG, Baranska W, Sawicki W, Koprowsky H: Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. Proc Natl Acad Sci USA 1971;68:353–357
2. Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C: Sperm cells as vectors for introducing foreign DNA into eggs: Genetic transformation of mice. Cell 1989;57:717–723
3. Gagne MB, Pothier F, Sirard M-A: Electrophoresis of bovine spermatozoa to carry foreign DNA in oocytes. Mol Reprod Dev 1991;29:6–15
4. Camaioni A, Russo MA, Odorisio T, Gandolfi F, Fazio, VM, Siracusa G: Uptake of exogenous DNA by mammalian spermatozoa: Specific localization of DNA on sperm heads. J Reprod Fert 1992;96:203–212
5. Francolini M, Lavitrano M, Lamia CL, French D, Frati L, Cotelli F, Spadafora C: Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. Mol Reprod Dev 1993;34:139–149
6. Chan PJ, Kalugdan T, Su BC, Whitney EA, Perrott W, Tredway DR, King A: Sperm as a noninvasive gene delivery system for preimplantation embryos. Fertil Steril 1995;63:1121–1124
7. Chan PJ, Seraj IM, Kalugdan TH, King A: Blastocysts exhibit preferential uptake of DNA fragments from the E6-E7 conserved region of the human papillomavirus. Gynecol Oncol 1995;58:194–197
8. Sarkar FH, Crissman JD: Detection of human papillomavirus DNA sequences by polymerase chain reaction. Biotechniques 1990;9:180–185
9. Barbosa M, Schlegel R: The E6 and E7 genes of HPV 18 are sufficient for inducing two stage in vitro transformation of human keratinocytes. Oncogene 1989;4:1529–1532
10. Ting Y, Manos, MM: Detection and typing of genital human papillomavirus. *In* PCR Protocols: A Guide to Methods and Applications, MA Innis, DH Gelfnad, JJ Sninsky, TJ White (eds). San Diego, Academic Press, 1990, pp. 356–367
11. Lauria A, Gandolfi F: Recent advances in sperm cell mediated gene transfer. Mol Reprod Dev 1993;36:255–257