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RESEARCH ARTICLE

Transfection of oocytes and other types of ovarian cells in rabbits after direct injection into uterine arteries of adenoviruses and plasmid/liposomes

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Transfection of oocytes should be avoided in somatic gene therapy. However, several viral vectors including adenoviruses can transfect zona-pellucida-free eggs in vitro. During early stages of development, oocytes of postnatal ovaries lack the zona pellucida. Therefore, they may be susceptible to gene transfer and unintended toxic effects. The purpose of this study was to see whether the injection of adenoviruses (1×10^{10} PFU) or plasmid (500 μg)/DOTMA:-DOPE (1:2) liposomes directly into uterine arteries in pregnant rabbits leads to transfection of oocytes and other types of ovarian cells. LacZ and herpes simplex virus thymidine kinase (HSV-TK) were used as transgenes. It was found that both adenovirus and plasmid vectors transfected oocytes at the primordial and primary follicle stage when they were not protected by the zona pellucida, whereas no transfection was seen in oocytes surrounded by the zona pellucida. Efficient transfection of corpus luteum and granulosa cells was also detected by adenoviral and plasmid vectors. Transfection of oocytes and other ovarian cells was verified by X-gal staining and laser microdissection, followed by PCR analysis. HSV-TK gene transfer, followed by ganciclovir treatment, led to destruction of a significant number of oocytes, whereas HSV-TK gene transfer alone did not lead to toxic effects. It is concluded that the presence of a high concentration of adenovirus or plasmid vectors via the uterine artery may lead to transfection of zona-pellucida-free oocytes and other ovarian cells.
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Keywords: *ovary; gene therapy; adenovirus; plasmid/liposome; LacZ; thymidine kinase*

Introduction

Transfection of oocytes is an undesirable event in somatic gene therapy. Germline transmission has been studied in female mice by injecting adenoviral vectors straight into ovaries or to their imminent proximity[1] or in male mice by intratestical or intraprostatic injections.[2,3] In these studies no germline transfection was detected. Neither did adenoviruses nor DNA-DOTAP complexes administered via intravascular or periadventitial routes affect germ cells.[4–7] In an *in vitro* study, Gordon found strong evidence that adenoviruses cannot infect oocytes and that the risk of germline transduction with such vectors is very low.[1] However, Chan *et al* produced transgenic rhesus monkeys by injecting pseudotyped replication-defective retroviruses into the perivitelline space of mature rhesus oocytes,[8] and Tsukui *et al* have reported that adenovirus vectors may transfect single cell mouse embryos *in vitro*.[9] These experiments raise the possibility that the presence of foreign DNA in the reproductive tract may constitute a germline hazard or even carry a risk for foreign gene integration.

We have previously studied prospects for the treatment of pregnancy toxemia using placental gene therapy.[10] Angiographically guided uteroplacental gene transfer with adenoviruses and plasmid vectors in rabbits resulted in a very high local transfection efficiency of placental trophoplastic cells. This led us to investigate whether the same approach would also affect germline cells. It was found that oocytes which lack the zona pellucida were affected by direct injection of vectors into uterine arteries.

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Results

Oocytes at every developmental stage and corpora lutea can be found in ovaries of pregnant rabbits. In maturing follicles, the oocyte is surrounded by the zona pellucida glycoprotein layer, granulosa cells and theca cells which protect oocytes from systemic factors (Figure 1). During ovulation, the mature follicle ruptures but the oocyte is still surrounded by zona pellucida and granulosa cells. The purpose of our study was to see whether direct injection of adenoviruses or plasmid/liposomes into arteria uterina in pregnant rabbits led to transfection of

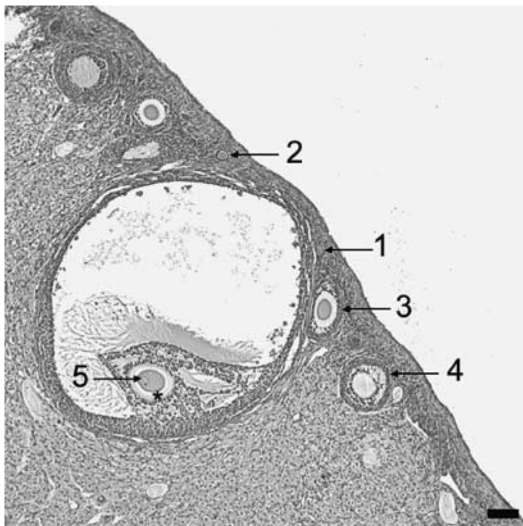

Figure 1 Histology of rabbit ovary. All follicular maturation stages are present in pregnant rabbit ovaries: primary oocyte in a primordial follicle (1), secondary oocytes in primary (2), secondary (3), antral (4) and Graafian follicles (5). *: Zona pellucida is shown around oocytes in the secondary and Graafian follicle. Hematoxylin–eosin staining. Scale bar 100 μm.

Table 1 Transfection efficiency in different types of ovarian cells with lacZ vectors

Cell types	*Adenoviruses*	*Plasmid/liposome complexes*
Oocytes	+	++
Granulosa cells	+++	++
Theca cells	++	+
Corpus luteum	++++	+
Stromal cells	++	+

+weak (<1%).++moderate (<10%).+++high (<50%).++++very high (<90%).

ovarian cells. In the first set of experiments, 1×10^{10} PFU nuclear-targeted lacZ adenoviruses (AdvlacZ) or 500 μg lacZ plasmid/liposomes (DOTMA:DOPE 1:2) were injected under X-ray guidance directly into uterine arteries. Transfection rate 3 days after the gene transfer was highest with adenoviruses, which led up to 90% efficiency in luteal cells, followed by up to 50% transfection efficiency in granulosa cells. Only <5% of the thecal cells were transfected. Transfected oocytes were also detected, with a transfection efficiency of 1 ± 1% (Table 1 and Figure 2a–c). In ovary samples taken from lacZ plasmid/liposome-treated animals, single granulosa and stromal cells were transfected. However, transgene expression was noted in 9 ± 3% of primary oocytes.

We next wanted to see whether transgenes with powerful pharmacological effects could alter ovarian histology or oocytes. The HSV-TK transgene was used for this purpose since the transgene itself does not lead to any effects in the transfected tissue but produces cytotoxic nucleotide analog which kill target cells after i.v. administration of ganciclovir.[11] The HSV-TK/ganciclovir system is also widely used in human gene therapy for malignant diseases.[12] It was found that both Adv-HSV-TK and HSV-TK plasmid/liposome vectors followed by ganciclovir treatment led to morphological changes in oocytes (Figure 2e and f). Damaged oocytes were in the primordial and primary follicle stage. Changes were detected in 28 ± 13% of all HSV-TK oocytes in plasmid/liposome-treated ovaries. In AdvHSV-TK-treated animals, the number of primordial follicles was diminished to one-third compared with the number of follicles in control samples and the morphology was abnormal in 23 ± 11% of the remaining oocytes. The number of affected oocytes was higher than the transfection efficiency observed after lacZ transfections which may be due to a bystander effect of the HSV-TK/ganciclovir system in which toxic nucleotide analogs can diffuse from transfected cells to nontransfected neighboring cells.[13] No morphological changes were found in HSV-TK-transfected ovaries when ganciclovir treatment was not given confirming that the vectors themselves did not cause the changes (Figure 2d).

Transfection of oocytes, granulosa cells and stromal cells by HSV-TK vectors was verified by laser microdissection. This technique allows precise isolation from histological sections of desired cell types which were then subjected to PCR analysis. The presence of HSV-TK transgene could be detected in primordial oocytes (Figure 3).

Discussion

According to our study, gene therapy may have effects on ovaries and zona-pellucida-free oocytes. The evidence comes from three sources. Firstly, some oocytes were positive for nuclear-targeted lacZ expression after the gene transfer. Secondly, expression of the HSV-TK transgene was detected in the primary oocytes obtained from ovaries by laser microdissection. Thirdly, the effect of the HSV-TK gene transfer and ganciclovir treatment was verified histologically. Positive X-gal staining indicated a transfection efficiency after AdvlacZ gene transfer in oocytes of ≈ 1%, whereas the effect of the HSV-TK gene transfer and ganciclovir treatment was seen in 26 ± 13% of the oocytes. The marked difference in the results obtained between lacZ and HSV-TK/ganciclovir treatments may be due to the bystander effect whereby toxic nucleotide analog diffuse from transfected cells to nontransfected cells:[13] transfection of granulosa cells by the HSV-TK gene probably contributed to the death of oocytes. In addition, PCR showed clear evidence for the transfection of individual oocytes obtained from ovaries by laser microdissection. Previous studies have shown that zona pellucida prevents transfection of oocytes *in vitro*.[8,9] This was also shown in our study, although the bystander effect of the HSV-TK gene transfer combined with ganciclovir treatment also affected oocytes surrounded by zona pellucida.

In somatic gene therapy, possible germline alterations caused by foreign genes should be avoided. The frequency of endogenous insertional mutations has been evaluated by Kazazian who concluded that although the desired number of insertions into the genome from exogenous agents would ideally be zero, the endogenous frequency of insertional mutations should be considered when estimating the potential risks of gene therapy on germ line.[14] He also comments that the occasional insertions of foreign DNA may not be as deleterious as

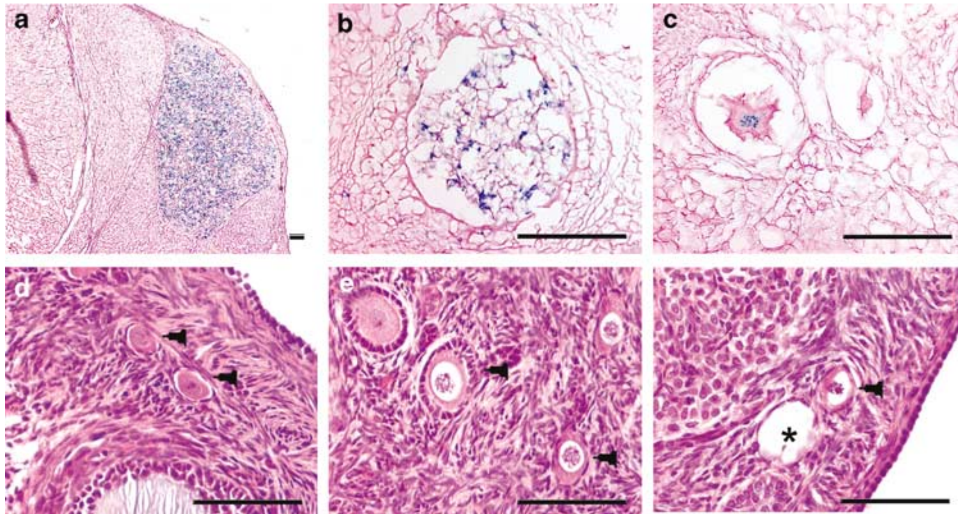

Figure 2 Transgene expression in ovarian cells. X-gal staining for nuclear-targeted β-galactosidase activity after gene transfer into uterine artery using either plasmid/liposome (500 μg) or lacZ adenoviruses (1.0×10^{10} PFU) (a–c). Blue stain indicates transfected cells. (a) AdvlacZ expression in corpus luteum. (b) Adenovirus transduced granulosa and theca cells in an antral follicle. Oocyte is not shown in the section. (c) LacZ Plasmid/liposome expression in an oocyte. All transduced oocytes were in primordial or early maturating stages and did not have zona pellucida. Hematoxylin–eosin staining after thymidine kinase gene transfer into uterine artery (d–f). (d) Normal rabbit ovary and oocytes (arrowheads). (e) Oocytes (arrowheads) in primordial follicles after plasmid/liposome-HSV-TK gene transfer and ganciclovir treatment. (f) Oocytes (arrowhead) in primordial follicles after AdvHSV-TK gene transfer (1.0×10^{10} PFU) and ganciclovir treatment. *: Vacuole indicating an apoptotic oocyte. Scale bars 100 μm.

has originally been expected. Although the present study did not test for germline transmission of the vectors, the results clearly show that female germ cells can be transduced with adenovirus and plasmid vectors and that the expression of the exogenous gene may cause changes whether or not it has been integrated into the germline. HSV-TK gene therapy has been widely used for the treatment of malignant diseases.[11,12] As shown here, the expression of the HSV-TK gene followed by the ganciclovir treatment may affect the number of oocytes, and in the worst case scenario may cause infertility. In addition to the germ cell production, the reproductive tissue also has important endocrinological functions, and the reduction in the number of hormone secreting cells caused by the HSV-TK treatment may alter hormonal balance of the treated patient. However, it should be recalled that current treatments for cancer with chemotherapy and radiation therapy also possess significant risks to ovaries and germline cells.

We have shown that zona-free oocytes from primordial follicles can be transfected *in vivo* using intravascular gene transfer with adenoviruses and plasmid/liposome vectors. Previous animal studies have failed to show transfection of oocytes with adenoviral and lentiviral vectors, or with DNA–liposome complexes.[1,4–7,15] One reason for this may be differences in the biodistribution of vectors in rodents and rabbits. Also, time of maturation of the follicles and the formation of zona pellucida is much shorter in rodents than in larger mammals,[16] which may at least partly explain the differences between our results and previous findings obtained from rodent studies. It is concluded that a high concentration of adenoviral or plasmid/liposome vectors in uterine arteries may lead to transfection of zona-pellucida-free oocytes and other ovarian cells.

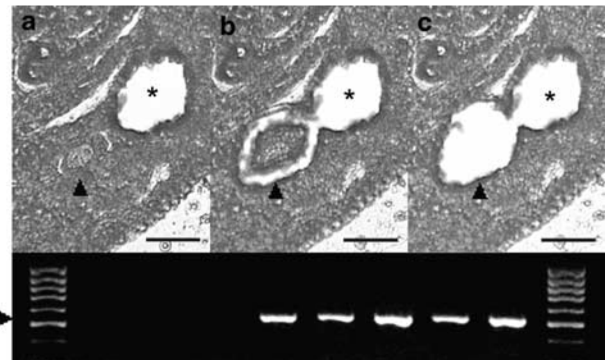

Figure 3 Detection of HSV-TK transgene from laser microdissected ovarian cells by PCR analysis. Dissection of individual primordial oocytes from eosin counterstained sections of rabbit ovary (a–c). (a) Primordial oocyte before laser microdissection (arrowhead) and a hole in the place of dissected oocyte (asterisk). (b) Laser cutting line around the oocyte (arrowhead). (c) A hole from dissected oocyte (arrowhead). Scale bars 50 μm. PCR of HSV-TK from microdissected ovarian cells (d). (1) water control; (2) oocyte control from AdvlacZ samples; (3) granulosa cell control from AdvlacZ samples; (4) HSV-TK oocyte; (5) HSV-TK granulosa cell; (6) HSV-TK luteal cell; (7) HSV-TK stromal cells; (8) HSV-TK plasmid control. Specific PCR product is 483 bp, as indicated by the arrow.

Materials and methods

Adenovirus and plasmid–liposome vectors

All vectors used for the study were human clinical-grade vectors containing HSV-TK gene or nuclear-targeted LacZ gene under a CMV promoter.[12] Replication-deficient adenoviruses (Ad5 serotype; E1-, partial E3-deletion) were produced in 293 cells, concentrated by ultracentrifugation and characterized with titer assay, Southern blotting, E1/E2 selective PCR and cytopathic

effect assay on A549 cells.[4,17] Plasmid vectors were produced in *E. coli* and purified with Qiagen method.

Animal model

Gene transfer was made to uterine arteries of pregnant New Zealand White rabbit does (n=18: weight 4.0–6.5 kg; gestational age 14–28) using catheterization. Animals were obtained from Finnish National Experimental Animal center. Rabbit does were anesthetized with Ketamine (10 mg/kg i.m. and i.v.) and promazinum hydrocloridium (0.5 mg/kg i.m. and i.v.). In addition, halothane was used during the catheterization. Under fluoroscopical control a 4 Fr. diagnostic catheter was positioned into the orifice of the uterine artery. In some cases, selective catheterization of the uterine arteries was done with a 2.1 or 3.0 Fr. Microcatheter (Tracker-18. Target Therapeutics). A total of 1.0×10^{10} PFU adenovirus (n=11) or 500 μg plasmid/DOTMA:DOPE liposome complex (1:2) (n=5) was injected into both uterine arteries in the final volume of 2.5 ml. Rabbits treated with LacZ gene transfer (AdvLacZ: n=7; lacZ plasmid/liposome: n=3) were killed 3 days after the gene transfer. HSV-TK-treated animals (AdvHSV-TK: n=4; plasmid/liposome HSV-TK: n=4) were given 25 mg/kg/day ganciclovir (Cymevene, F. Hoffman-La Roche, Basel, Switzerland) on days 3–5 after the gene transfer. Eight days after the gene transfer, the animals were killed and the samples were collected via laparotomy.

Histology

After killing, ovaries were removed and part of the ovaries were immersion-fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) (pH 7.4) for 30 min, rinsed for 2 h in PBS, embedded in OCT compound (Miles) and stored at −70°C.[18] Part of the ovaries were immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.5) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin.[18] Hematoxylin–eosin staining was used for general morphologic evaluation. Histology was analyzed using Image-Pro Plus software with Olympus AX70 microscope (Olympus Optical, Japan).

X-gal staining

OCT embedded ovaries were cut into 12 μm sections, stained with X-gal reagent (100 mg/ml; 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactoside to 10 ml dimethyl formamide) diluted 1:100 with X-gal solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$) and incubated in the dark at 37°C for 8–18 h.[19] Thereafter, sections were rinsed with PBS, dehydrated with rising ethanol concentrations and counterstained with Meyer's carmalum. More than 2000 nuclear-targeted X-gal-positive cells were counted in the lacZ-transfected ovaries for the calculation of the transfection efficiency in different types of ovarian cells.

Laser microdissection

Sections (4 μm) were cut from paraffin-embedded tissue samples. Briefly, sections were deparaffinized twice in xylene for 5 min, rehydrated in 100% ethanol, 95% ethanol, 70% ethanol and sterile water each for 30 s, stained with hematoxylin for 30 s, rinsed in water and stained with eosin for 30 s, and then dehydrated in graded ethanol and xylene. A total of 50 individual oocytes were dissected using Leica DM LMD Microdissection system. Using laser beam, tissues were collected directly into 0.5 ml microtubes containing 50 μl lysis buffer (0.5% Tween 20, 1 mM EDTA, 50 μM Tris pH 8.5, 0.5 μg/μl Proteinase K) and incubated at +37°C overnight.[20] Following incubation, samples were extracted once with chloroform–phenol and DNA was precipitated from the aqueous phase with 200 μl of ethanol in the presence of 10 μg of glycogen carrier (MBI Fermentas), and resuspended into 10 μl of dH_2O.

PCR

For HSV-TK PCR, 5′ primer 5′-AAA TGG GCG GTA GGC GTG TA-3′ was from the CMV promoter and 3′ primer 5′-AGG CGG TGT TGT GTG GTG TA-3′ was from the HSV-TK coding region. Each reaction mixture contained 20 pmol of primers, 200 μM of deoxynucleotide triphosphates (MBI Fermentas), 1.5 mM $MgCl_2$, 1 U of dynazyme polymerase (Finnzymes, Finland) and $1 \times$ PCR reaction buffer (Finnzymes, Finland). PCR with initial denaturation (+94°C for 4 min) was followed by 10 cycles of +94°C for 30 s, +66°C for 30 s and +72°C for 60 s, and 30 cycles of +94°C for 30 s, +62°C for 30 s and +72°C for 60 s with the final extension of 10 min at +72°C. The length of the amplified fragment was 483 bp.

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