No evidence of germ-line transmission by adenovirus-mediated gene transfer to mouse testes

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Objective: To investigate the risk of germ-line transmission of vector sequences after in vivo adenovirus-mediated gene transfer to mouse testes and to discuss whether an adenovirus vector could be used in the future to treat male factor infertility.
Design: Experimental animal study.
Setting: Laboratory research setting in the Department of Nephro-urology at Nagoya City University Graduate School of Medical Sciences in Japan.
Animal(s): Eight-week-old B6C3F1 mice.
Intervention(s): Adenovirus vector carrying a *LacZ* transgene as a marker was injected into the interstitial space (intratesticular injection) or seminiferous tubules (intratubular injection) of the mouse testis.
Main Outcome Measure(s): An assessment by polymerase chain reaction (PCR) and histological analyses of the proportion of adenovirus vectors administered into the testis that can infect epididymal sperm and transmit to fetuses derived from these males 3, 7, 14, 28, and 35 days after intratesticular or intratubular adenovirus injection.
Result(s): No PCR signal was identified in genomic DNA extracted from the epididymal sperm of all mice on each day after intratesticular or intratubular adenovirus injection. On reverse transcriptase (RT)-PCR analysis of mRNA isolated from fetuses derived from these males on each day after intratesticular or intratubular adenovirus injection, no fetuses had amplified products, although about 30% of the fetuses generated by microinjection into fertilized eggs had LacZ transcripts. On histochemical staining, no two-cell and 12.5 d.p.c. fetuses showed β-gal activity. These sperm and fetus studies showed that adenovirus-mediated gene transfer to the testis does not cause infection of or transmission to the germ line or fetuses.
Conclusion(s): The risk of germ-line transmission after adenovirus-mediated gene transfer to the testis is extremely low, and this method can be exploited in the future for the treatment of male factor infertility. (Fertil Steril® 2008;89:1448–54. ©2008 by American Society for Reproductive Medicine.)

Key Words: Germline transmission, adenovirus, gene transfer, testis

Testicular sperm extraction (TESE) with intracytoplasmic sperm injection (ICSI) has opened new treatment perspectives for patients with male factor infertility and is now a first-line treatment, especially for azoospermic patients (1). Many men who were previously thought to be irreversibly infertile have the potential to initiate biologic pregnancy with the possibility of paternity. However, some problems with TESE-ICSI have not been solved, and fertilization is considered to be better in vivo than in vitro in male factor infertility. If spermatozoa exist in the testis of infertile men, logically there is a possibility of paternity using TESE-ICSI. On the other hand, if there are no spermatozoa in the testis in these patients because of pathological maturation arrest or because of having Sertoli cells—only, they do not have paternity potential even if TESE-ICSI is conducted.

Gene therapy is used to introduce genes into cells to cure a defect or to slow the progression of inherited or acquired disease. Many methods and techniques for in vivo gene transfer have been developed, and some have already been applied in clinical trials. Clinical trials designed to maximize scientific information about gene delivery and the potential toxic effects of therapy provide a basis of knowledge for future therapeutic strategies.

Some causes of human male factor infertility may be associated with testicular somatic cells including Sertoli and Leydig cell dysfunction. Several possible genes may be essential for Sertoli and Leydig cell function to support normal spermatogenesis. Testicular gene therapy for these cells as target cells may be useful to treat male factor infertility in the future, especially for those men who have no spermatozoa in their testes at all because of pathological maturation arrest or Sertoli cells only. For clinical application, it is important to understand not only the effectiveness but also the safety

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and control of transferred gene expression. Although several methods for gene transfer to testicular cells and sperm in animals have been reported (2–6), adenovirus vector is considered to be one of the best methods for transfecting testicular somatic cells because of its effectiveness as well as low testicular damage (7); however, we have to confirm that there is no possibility of germ-line transmission by adenovirus-mediated gene transfer to the testis to realize future clinical trials of gene transfer for male factor infertility patients because gene therapy is acceptable only as long as germ-line transmission can be avoided (8). In this study, we directly injected DNA into mouse testes in vivo to transfect testicular cells with foreign DNA using adenovirus vector and analyzed whether foreign DNA can transmit to the germ line and fetuses, and we discuss the potential of gene therapy for male factor infertility.

MATERIALS AND METHODS

Animals

Male B6C3F1 mice (Japan S.L.C., Hamamatsu, Japan), 8 weeks of age, were individually housed at a room temperature of approximately 23°C with alternating 12-hour periods of light and dark. These studies were performed in accordance with the Guidelines for the Care and Use of Experimental Animals and were approved by the Animal Care Committee and Institutional Review Board of the Nagoya City University Graduate School of Medical Sciences.

Preparation of Adenovirus Vector

A replication-incompetent human adenovirus serotype 5 contained two deletions (E1 and E3 deletions) and was constructed such that the transgene (*CAG-LacZ*) was driven by the chicken β-actin promoter to promote overexpression of the downstream target gene (*LacZ*), as described elsewhere (7). *LacZ* (approximately 3 kb in size) contains a sequence of the E. coli *β-galactosidase (β-gal)* gene. High-titer viral stock was prepared, and quick plaque-forming unit (PFU) assays were performed using 293 human embryonic kidneys cells. The stock used in this study was prepared at a concentration of 1×10^9 PFU/mL sterile phosphate-buffered saline (PBS) with 0.68 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

Intratesticular Injection and Intratubular Injection

Male mice were anesthetized with pentobarbital. A scrotal incision was made, and testes were delivered through the wound. For intratesticular injection, 20 μL per testis of adenovirus solution, to which 0.04% Trypan blue dye had been added to monitor the accuracy of the injection, was injected into the interstitial space of the bilateral testes (n = 85) using a 30-gauge needle and a 1.0-mL disposable syringe. For intratubular injection, bilateral testes were pulled out and exposed under a dissecting microscope. A small incision was made in the tunica, and then 20 μL per testis of DNA solution with 0.04% Trypan blue dye was injected into the seminiferous tubules (n = 85) using injection glass pipettes. Injections were made at three sites in each testis. At various times after injection (3, 7, 14, 28, and 35 days), 25 mice per injection method were sacrificed, and bilateral testes and epididymides were removed to investigate the presence of vector DNA in the testis and sperm by polymerase chain reaction (PCR); the others were mated with untreated female mice to investigate the presence of vector DNA in their fetuses.

Mating with Female Mice

Each treated male mouse was housed with four 8-week-old untreated females per cage for 1 day. To time pregnancies, the day of the appearance of a vaginal plug was taken as 0.5 d.p.c. To clarify fetus transmission of the *CAG-LacZ* gene, pregnant females were killed at several pregnancy stages, and two-cell and 3.5, 7.5, and 12.5 d.p.c. fetuses were investigated. Transgenic carriers were identified by reverse transcriptase (RT)-PCR analysis and β-gal expression.

Isolation of Testicular and Epididymal Sperm DNA and Genomic PCR

After the isolation of testicular and epididymal sperm DNA from each adenovirus-injected male mouse, PCR analysis was performed to amplify a 332-bp fragment of *β-gal* using sense primer 5′-GCCGAAATCCCGAATCTCTATC-3′, corresponding to nucleotides 907–928 in the *β-gal* gene and antisense primer 5′-GGCTTCATCCACCACATA-CAGG-3′, corresponding to nucleotide 1218–1239 in the *β-gal* gene. An aliquot of the product was size fractionated by electrophoresis through a 3% agarose gel containing ethidium bromide and visualized by exposure to ultraviolet radiation.

RT-PCR Analysis of β-gal mRNA in Fetuses Obtained after Injection into Testis and Mating with Females

For RT-PCR detection of LacZ transcripts, total RNA was extracted from fetuses (3.5, 7.5, 12.5 d.p.c) using an RNAqueous Kit (Ambion, Austin, TX) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized with 5 μg of total RNA using an oligo(dT) primer and then used for RT-PCR. A 600-bp fragment was amplified for 30 cycles with the following primers: forward, CGCCT TGCAGCACATCCCCCTTTC, complementary to nucleotides 76–99 of the *β-gal* gene; reverse, CACGCAACTCG CCGCACATCTGAACT, complementary to nucleotides 729–754 of the *β-gal* gene.

Real-Time Quantitative RT-PCR

TaqMan PCR was performed to quantify the expression level of LacZ transcripts in fetuses (12.5 d.p.c) produced by mating with males 3, 7, 14, 28, and 35 days after injection using the Applied Biosystems PRISM 7700 sequence detection system (Applied Biosystems, Branchburg, NJ). The primers and probes were designed with PRIMER-EXPRESS software