Fertility After Homologous Prepubertal Testis Transplantation in the Dog

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ABSTRACT
Canine models of hereditary human diseases are widely used throughout the biomedical community, particularly when no suitable rodent model exists. In several models, the homozygote dogs die prior to puberty, or have substantially reduced fertility. Prepubertal transplantation of the testes was used to propagate the genotype of a mutant dog that would not otherwise have survived until puberty. The transplant recipient remained fertile 7 years postoperatively. To begin determining the factors necessary for successful function in testis transplants, prepubertal dogs that were dog leukocyte antigen (DLA) identical and disparate were examined for fertility and compared to the original transplant recipient as well as unoperated and sham-operated dogs. Immunosuppression was maintained with cyclosporine (CyA) and prednisone in the immediate postoperative period and CyA alone thereafter. The DLA-identical dogs demonstrated initial acceptance of the transplant, whereas one of two underwent chronic rejection. Both DLA-disparate dogs had subacute rejection prior to sexual maturity. These results demonstrate that homologous transplantation of prepubertal testes can be an effective method to preserve genotype in DLA-identical dogs. This model may also be useful for studying testis development and immunobiology.

ANIMAL models of inherited human disease occupy a vital place in the national research strategy. Although the advent of genetically engineered mutant mice has resulted in a tremendous increase in the use of mouse models of inherited human disease, canine models are still utilized throughout the biomedical community. Several canine models of hereditary diseases have no equivalent mouse mutation, and research effort is generally focused on affected homozygotes. These colonies can be difficult to maintain in an efficient manner owing to two primary obstacles. The first problem is a lack of sufficient numbers of affected homozygote animals. In several models, affected homozygote dogs die prior to puberty and, therefore, cannot be bred to produce additional homozygotes. Breedings between heterozygotes will result in an average of only 25% of offspring carrying the homozygote genotype. This is particularly problematic for dogs and other larger species, such as primates, which have a significantly lower litter size than rodents. Though mice can have estrous cycles every 5
days, dogs have a maximum of two estrous cycles per year, further reducing the maximum number of homozygote dogs that can be produced by breeding two heterozygotes. Lack of sufficient numbers of affected homozygotes can result in substantial research study delays for investigators who rely on these animals.

The second major difficulty in canine breeding colony production is high per diem costs. The inability to breed affected homozygotes requires that large numbers of heterozygote breeders must be maintained in order to produce a sufficient number of affected homozygotes. Dog per diem rates can be up to 20 times higher than those of mice; therefore, colony managers typically maintain the minimum number of animals to produce the necessary number of offspring. Homologous prepubertal gonad transplantation from a homozygous donor to a normal littermate would allow a normal recipient to be bred, while passing on the genotype of the affected donor. The number of homozygotes produced can be increased by 100% over traditional heterozygote breeding.

We reported the initial case of successful prepubertal testes transplantation in the dog. Here we show that this animal remained fertile 7 years postoperatively and sired two litters. To begin the identification of factors that are necessary for success, dog leukocyte antigen (DLA) identical and disparate littersmates were used to determine whether DLA matching is required for successful transplantation. Results from these dogs were compared with the original transplant recipient as well as unoperated and sham-transplanted dogs. Animals were evaluated for serum testosterone, testicular blood flow, and semen quality.

MATERIALS AND METHODS

Animals

Each pair of donors, recipients, and control dogs were prepubertal hound littermates (Covance Research Products, Denver, Penn). Red blood cell (RBC) crossmatching for alloantibodies, RBC typing, lymphocyte crossmatching, and DLA typing at DRB and DRQ (class II) was performed by Midwest Animal Blood Services (Stockbridge, Michigan). Dogs receiving transplants were either DLA identical (n = 2) or DLA disparate (n = 2). Although the original transplant recipient was DLA-identical to its donor, it is hereafter referred to as the original recipient to avoid confusion. Animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility and in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee.

Transplant Surgery

The surgical procedure was performed as described previously. Briefly, the testicular artery, vein, and vas deferens were connected via an end-to-end anastamosis using 10-0, 10-0, and 7-0 nylon, respectively. Because of the length of time required to perform the procedure, only one testis was transplanted into each recipient. For the sham transplant (n = 1), one testis was removed and the other was transected and reattached via end-to-end anastamosis.

Immunosuppression

Although the interior of the testis is immunoprivileged, the transplanted vessels and vas deferens are not, so immunosuppressive therapy was used. Transplanted dogs were maintained on aspirin (40 mg PO q 24 hours) for 21 days for clot inhibition, and on prednisone (10 mg/kg PO q 24 hours) and cyclosporine (CyA) (15 mg/kg PO q 24 hours; Sandimmune, Novartis Pharma AG, Basel, Switzerland) continuously. Unoperated control dogs (n = 6) were maintained on CyA to determine whether CyA alone would have adverse effects on fertility.

Clinical Monitoring

The general health of the animals was monitored in addition to evidence of graft-versus-host disease and secondary infections due to immunosuppression. Dogs were also routinely examined for gingival hyperplasia due to chronic CyA administration.

Measures of Fertility

Serum testosterone was measured via radioimmunoassay (Emory University Hospital, Atlanta, Georgia, and Antech Diagnostics) at various time points before and after transplantation. Blood CyA levels were measured (Emory University Hospital). Semen samples were collected with the use of an artificial vagina from the original, sham, and control dogs prior to euthanasia. Semen from the successful DLA-identical dog was collected postmortem, as the dog would not submit to antemortem collection. Sperm counts were analyzed using a standard hemocytometer. Percentages of progressively motile and abnormal sperm morphology were assessed visually. Doppler ultrasound examination was performed at various time points after transplantation to monitor continued blood flow to the testes. Tissues were fixed in 10% formalin, and sections were stained with hematoxylin-eosin and Masson’s trichrome.

RESULTS

Clinical Monitoring

No evidence of graft-versus-host disease was detected in any dog. The only adverse effect from chronic CyA administration in some dogs was gingival hyperplasia, which was treated with azithromycin (40 mg/kg/d for 5 days).

Measures of Fertility

The original transplant recipient sired two litters of pups (n = 4 born and phenotyped per litter; n = 2 homozygotes per litter) and maintained normal serum testosterone levels for 213 weeks postoperatively, while the sham transplant maintained normal serum testosterone for its entire experimental period, 70 weeks postoperatively (Fig 1). One DLA-identical dog maintained normal serum testosterone until 63 weeks’ postoperation, when it was euthanized for chronic, debilitating intestinal cryptosporidiosis; the other DLA-identical animal had undetectable serum testosterone levels by week 48 (Fig 1). Both DLA-disparate dogs had serum testosterone levels below the level of detection 8 weeks’ postoperation (Fig 1). All four control dogs maintained serum testosterone levels within the range for normal intact dogs for 3 years after the start of treatment (Fig 2).
Semen samples for analysis were collected from the original and sham recipients, as well as from controls. The average total sperm output was $300 \times 10^6$, $280 \times 10^6$, and $410 \times 10^6$, respectively. No animal had more than 20% morphologically abnormal sperm, and the percent of progressively motile sperm ranged from 70% to 85%. All semen samples collected were within the normal range for fertile dogs. 

Unfortunately, the successful DLA-identical recipient would not submit to antemortem semen collection; therefore, only the presence of sperm from a smear of the epididymis could be confirmed.

Color Doppler ultrasound examination of the transplanted testes 2 weeks postoperation compared with a control demonstrated normal blood flow for the sham and the more successful DLA-identical dog, whereas one of the DLA-disparate animals showed very little blood flow to the testis (Fig 3). Histological comparisons of the original transplant recipient, unoperated controls, sham transplant, and the longer-term DLA-identical dog were almost indistinguishable, although the DLA-identical animal had slightly decreased cellularity of the basal levels of the seminiferous tubules (Fig 4). In addition to the decreased number of primordial germ cells observed, the dog demonstrated a lower level of sperm in the semen (Fig 4). Rejection of the testis demonstrated in one of the DLA-identical dogs, namely increased fibrosis and lymphocytic infiltration, was very similar to the rejection observed in the DLA-disparate animals (Fig 4).

Blood CyA levels were evaluated at a single postoperative time point for each dog. Values for the DLA-identical dogs were 252 ng/mL and 544 ng/mL and 198 ng/mL and 1345 ng/mL for the DLA-disparate animals. The original transplant recipient, sham-transplant recipient, and unoperated control dogs demonstrated a range from 300 ng/mL to 1013 ng/mL. Because levels did not differ between animals that rejected and those that did not, further examination of CyA levels was not conducted. The successful DLA-identical dog was euthanized at 63 weeks postoperation due to intestinal cryptosporidiosis, while the original recipient was euthanized at 7 years postoperation due to intestinal candidiasis that did not respond to treatment.

**DISCUSSION**

The original recipient was the first published case of a prepubertal canine testes transplant. One group performed testis transplants in adult dogs, but fertility was only evaluated by histology. Though our first transplanted recipient was fertile, the parameters necessary for successful prepubertal testis transplantation remained unknown. Although the original donor and recipient were not DLA typed prior to transplantation, retrospective testing revealed that they were DLA-identical. Subsequent transplants using DLA-identical dogs had mixed results, with one animal undergoing subacute rejection and one demonstrating testicular function. The original, sham, and DLA-identical transplant recipients all reached puberty, as demonstrated by serum testosterone levels consistently above 0.2 ng/mL, the minimum expected for an intact animal. An untreatable intestinal infection in the successful DLA-identical dog prevented it from surviving long enough to
become socially mature enough to breed with a female; however, it exhibited sperm production and normal serum testosterone levels. In contrast, the DLA-disparate dogs rejected the transplanted testis shortly after surgery. These results are consistent with other organ transplants in dogs, which indicate that success is increased in DLA-matched animals.\(^6\)–\(^9\)

Ultrasound examination can be used to monitor for viability of the testes after surgery, prior to puberty and consistent testosterone production, as differences in blood flow between successful and unsuccessful animals were readily apparent. Histologically, productive seminiferous tubules were detected in the unoperated control, original, sham, and successful DLA-identical recipients, while the DLA-identical dog that rejected was indistinguishable from the DLA-disparate animals. Immunohistochemistry and the presence of antibody development were not examined. Semen samples were consistent with the histological impression, in that dogs that demonstrated a proliferation of spermatagonia in the seminiferous tubules had sperm in the semen.

The use of more modern immunosuppressive drugs either alone or in addition to CyA may have increased success, as demonstrated in transplantation of other organs in dogs.\(^10\)–\(^15\) While the immunosuppressive therapy regimes in these studies were able to prevent rejection, these drugs can be three times more costly than CyA. If prepubertal gonad transplantation is to be used routinely in the management of canine breeding colonies, the immunosuppressive therapy must be both successful and cost-effective. The original and sham recipients, as well as unoperated controls, demonstrated that testicular function was unaffected by chronic CyA administration. Cyclosporine is neither nephrotoxic nor cytotoxic in dogs,\(^16\) eliminating concerns about renal toxicity seen in humans. The intestinal fungal infections observed in the original and successful transplant recipients were likely the result of long-term immunosuppression and illustrate the challenge of maintaining canine transplant recipients that cannot practically be kept under sterile conditions, which is routine for immunosuppressed mice.

The results of this study demonstrate that fertility and testicular function after prepubertal testis transplantation is possible using DLA-identical dogs. However, DLA matching is not sufficient for success as evidenced by rejection observed in one of the DLA-identical recipients. One
limitation may be that major histocompatibility testing for dogs consists of examining polymorphisms of only two genes.\textsuperscript{17–19} The role of minor histocompatibility antigens is unknown. The original transplant recipient came from a highly inbred colony (typical for mutant dog colonies) with an inbreeding coefficient of 0.42, whereas the DLA-identical hounds were from an outbred colony with an inbreeding coefficient of zero. Future studies will evaluate fertility in highly inbred DLA-identical testis transplant recipients to determine whether the degree of inbreeding is also critical for success, as opposed to DLA matching alone. The ability to transplant prepubertal testes may also help preserve valuable genotypes in other species, in which animals of the desired genotype cannot survive to puberty.

Fig 4. Testis with normal seminiferous tubules in an unoperated (A), sham transplant (B), original transplant (C), and successful DLA-identical transplant recipient with slightly reduced number of germ cells (D, H&E ×100). Semen smear from successful DLA-identical recipient (E, H&E ×400) demonstrating the presence of spermatozoa (arrows). Testis from rejected DLA-identical dog exhibiting fibrosis, lymphocytic infiltration, and degenerated seminiferous tubules (F, H&E ×100). Lymphocytic infiltration with degenerated seminiferous tubules (G, H&E ×100) and severe fibrosis between seminiferous tubules (H, Masson’s trichrome ×100) in a DLA-disparate recipient.
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REFERENCES
2. Pullium JK, Lin PH, Pinter MJ: Homologous transplantation
of prepubertal testes in the dog. Transplantation 72:552, 2001
3. Whitmore WF, Gittes RF: Intratesticular grafts: the testis as
an exceptional immunologically privileged site. Trans Am Assoc
Genitourin Surg 70:76, 1978
4. England GCW: Allen’s Fertility and Obstetrics in the Dog,
veterinary medicine. In Kirk RW (ed): Current Veterinary Ther-
survival of DLA-matched segmental small-bowel allografts in dogs.
Transplantation 56:1062, 1993
morphologic, and functional evaluation of long-term-surviving
beagle lung allograft recipients treated with lethal total-body
irradiation, autologous bone marrow, and methotrexate. Trans-
plantation 44:179, 1987
chimerism in DLA-identical littermate dogs given sublethal total
body irradiation before and pharmacological immunosuppression
immunosuppression of FK 506 in canine lung allo-transplantation.
with FK-506 and cyclosporine for canine lung allotransplantation:
immunosuppressive effects and blood trough levels. J Heart Lung
Transplant 12(6 Pt 1):941, 1993
liver allograft survival by a novel immunosuppressant, FTY720:
effect of monotherapy and combined treatment with conventional
drugs. Transplantation 69:235, 2000
analag, MNA-715, plus cyclosporine reduces renal allograft rejec-
therapy with FTY720 prolongs allograft survival after canine
kidney transplantation. Transplant Proc 31:2783, 1999
study of a novel immunosuppressant, FTY720, with the canine
renal allograft transplantation model. Transplant Proc 31(1–2):
1208, 1999
Veterinary Therapy, Vol. X. Philadelphia: W.B. Saunders; 1989,
p 513
17. Wagner JL, Burnett RC, Storb R: Molecular analysis of the
DLA DR region. Tissue Antigens 48:549, 1996
analysis and polymorphism of the DLA-DQB genes. Tissue Anti-
gens 52:242, 1998
19. Wagner JL, Burnett RC, Storb R: Organization of the
canine major histocompatibility complex: current perspectives.
J Hered 90:35, 1999