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Rat-to-Chinese tree shrew heart transplantation is a novel small animal model to study non-Gal-mediated discordant xenograft humoral rejection

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Abstract

Since α -1,3-galactosyltransferase knockout (GalT-KO) pigs became available, there has been an increasing interest in non-Gal natural antibody (nAb)-mediated xenograft rejection. In order to better understand mechanisms of non-Gal nAb-mediated rejection, a simple small animal model without gene manipulation would be extremely valuable. Here, we tested whether the Chinese tree

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shrew (CTS), which is a small-sized mammal that is phylogenetically close to primates, could serve as a model for discordant xenograft rejection.

Methods

Study 1: Expression of α -Gal antigens in hearts and kidneys of CTSs and rats was assessed by IB4 lectin binding. Presence of anti-Gal and anti-non Gal IgM and IgG nAb in CTS sera was tested by FACS using Gal+ and GalTKO PBMC as well as BSA-ELISA.

Study 2: Rat hearts were transplanted into CTS recipients (group 1, n=7); and CTS hearts were transplanted in rats [n=10; Seven received no immunosuppression (group 2) and three received FK506+leflunomide (group 3)].

Results

Study 1: Both CTSs and rats had α -Gal expression in hearts and kidneys. ELISA showed CTSs do not have anti-Gal nAb and flow cytometry indicated CTSs have anti-non Gal IgM and IgG nAb in serum.

Study 2: Rat hearts in CTSs were uniformly rejected within 35 minutes, while CTS hearts in rats continued beating until day 5 without immunosuppression, and up to day 8 with immunosuppression.

Conclusion—Rat-to-CTS is a discordant while in the reverse direction, CTS-to-Rat is a concordant xenotransplant model. CTSs are valuable small animals to study mechanisms and strategies to avoid non-Gal nAb mediated xenograft rejection.

Keywords

Chinese tree shrews; Xenotransplantation; Cardiac transplantation; Animal model; Xenograft rejection

Introduction

Although swine are ideal donors for clinical xenotransplantation, hyperacute rejection in primates mediated by preformed natural antibodies (nAbs) targeting alpha-1,3-galactosyl antigens (a-Gal) prevented the progress of xenotransplantation (1, 2) in the 1990s. A major breakthrough in xenotransplantation was the production of α -Gal transferase knockout (GalT-KO) pigs in 2002 (3–5) which eliminated what was thought to be the most difficult barrier to overcome in xenotransplantation. Since then, non-Gal nAbs have become one of the major research topics in this field (6, 7). Non-Gal nAbs are primarily studied either in preclinical large animal models, or in GalT-KO mouse models (8, 9) However, in order to better understand mechanisms of non-Gal nAb-mediated rejection, a simple small animal model without gene manipulation would be extremely valuable.

Chinese tree shrews (CTS), a wild animal found in Southeast Asia part of the Tupaiidae family, has been widely used in research for several decades (9). Because of a variety of unique characteristics including their closer affinity of genome sequencing to non-human primate (NHP), a body size that is similar to rats, a short reproductive cycle, small space needed for housing, and low purchase costs, tree shrew were considered an ideal

experimental animal in research models of hepatitis virus (10), opiate addiction (11) and stage II pharmacological safety testing (12). A recent study has investigated the genome of the CTS suggesting that the tree shrew nervous and immune systems are phylogenetically similar to those of primates (13). This observation makes tree shrew a potential model of immunological research.

In this study, we investigated whether CTS could serve as a model for discordant xenograft rejection. Our data indicate that Rat-to-CTS and CTS-to-Rat are a discordant and a concordant model, respectively.

Material and Methods

Animals

Female Lewis rat, 6–8 weeks of age, weighing 100–120g were purchased from Vital River Laboratory Animal Co. Ltd (Beijing, China) and 3-month old female CTSs weighing 95–110 g were obtained from the Animal Experimental Centre of Kunming Medical University, Unnan, China.

Anesthesia

Anesthesia in donor CTS was induced with intraperitoneal injection of ketamine at 70 mg/kg and atropine at 0.03 mg/kg, followed by chloral hydrate at 200mg/kg for CTSs and at 450mg/kg for Lewis rats to maintain anesthesia.

Heart transplantation

Heterotopic heart transplantation was performed using inferior vena cava and abdominal aorta as previously described (14–16). Graft survival was monitored by palpation daily, and rejection was defined as the loss of palpable cardiac contractions.

Histopathology and Immunohistochemistry

After the grafts stopped beating, all the animals in each group were sacrificed and the heart grafts were harvested. One half of graft was used for histological evaluation and remaining half was used for immunofluorescent assay. Grafts prepared for histological analysis were fixed in 10% paraformaldehyde and then embedded in paraffin. Transventricular tissue sections (3µm thick) were stained with hematoxylin and eosin (H&E). Grafts prepared for immunofluorescence analysis were stored in a –80°C freezer until analysis. Transventricular tissue sections (3 µm thick) were stained with anti-human IgG, IgM and complement C3. Presence of αGal antigens in naïve CTS and rat were tested on paraffin-embedded kidney sections embedded with an αGal-specific lectin GSIB-4. Briefly, tissue sections were deparaffinized and hydrated through xylene and graded alcohol series and rinsed for 5 minutes in tap water; then treated with 0.1% pepsin at 37°C for 30 minutes and rinsed for 5 minutes in tap water. Non-specific binding block was achieved incubating sections with H₂O₂ for 30 minutes at room temperature. After a 5 minute rinse in tap water, biotinylated lectin was applied at approximately 20 µg/ml in PBS and sections were incubated for 60 minutes at room temperature. Sections were rinsed for 5 minutes in tap water and incubated with horseradish peroxidase for 30 minutes at room temperature. After a wash with TPBS

DAB staining for 2–10min was performed, and sections were then rinsed in tap water, and counterstained with hematoxylin as previously described (17).

Pig peripheral blood mononuclear cells

To detect the presence of anti-pig (anti-Gal+ and anti-non-Gal) antibodies in the serum, peripheral blood mononuclear cells were isolated from wild type (WT) and GTKO pigs (pPBMC; TBRC, MGH, MA, USA), as previously described [18].

Flow cytometry

Binding of CTS serum IgM and IgG to Gal+ and GalTKO pig PBMCs was assessed using a FACSort machine (Beckton Dickinson, San Jose, CA).

ELISA

CTS serum anti-Gal antibodies were evaluated using the CytoScreen ELISA kit (BioSource International) as follows (18). Plates were coated with 5µl/ml BSA-Gal (ET 14–43 N-value 36) overnight, washed 3 times, blocked with fish gelatin for 1 hour at 37°C. After 3 washes, serum from CTS was added and the plate was incubated for 1 hour at 37 °C; plates were washed 3 times and Rabbit anti-Human IgM-HRP was added (Dako Catalog #P0215) for 1 hour at 37 °C. After 3 washes, ABTS was added for 15 minutes then the reaction was stopped with 1% SDS. Plates were read using a microplate reader at 405 nm. Each sample was analyzed in triplicate wells.

Results

1. Assessment of anti-Gal and non-Gal nAb in CTS

Since CTSs are similar in size to rats but are phylogenetically closer to primates, we first assessed whether (1) CTSs have anti-Gal or anti-non-Gal antibodies and whether (2) hearts and kidneys of CTSs express αGal antigens.

We first assessed if CTSs have anti-Gal nAb by αGal-BSA ELISA assay. As a positive control we used baboon sera, which was strongly positive for anti-Gal IgM; no anti-Gal IgM nAbs were found in CTS sera similarly to the negative control (Figure 1A). As CTS did not have anti-Gal IgM nAb, we next assessed the presence of anti-non-Gal IgM and IgG nAbs in CTS sera by FACS (Fig. 1B). FACS data demonstrated that CTSs sera were strongly positive for both IgM and IgG to GalT-KO PBMCs (Fig 1B left and right). Mean fluorescence intensities of both non-Gal IgM and IgG nAbs in CTS sera were similar.

We further confirmed the presence of αGal antigens in naïve hearts and kidneys of CTS antigens using IB4 lectin in an immunohistochemical assay (Fig 2). MGH GalT-KO miniature swine cardiac tissues were used as negative control and regular MGH miniature swine cardiac tissues and rat cardiac tissue were used as positive controls of αGal expression. No IB4 positive cells were seen in GalT-KO miniature swine hearts (Fig. 2A-a), while αGal expression were diffusely observed on vascular endothelial cell as well as myocardial interstitial cells in the naïve MGH miniature swine (Fig 2A-b). Rat hearts also express αGal (IB4 positive), however, the staining with IB4 was less obvious (Fig 2A-c)

than that seen in swine hearts. Despite the fact that CTSs are phylogenetically close to primates, cardiac endothelial cells were positive for α Gal antigens, although in a more “patchy” pattern than was observed in rat hearts (Fig 2A-d). Rat kidneys expressed high levels of α Gal antigens at the brush border membrane of proximal tubules, on peritubular capillaries as well as on glomerular endothelial cells (Fig 2B-a). Staining patterns of IB4 in CTS were scattered in CTS kidneys as in the CTS hearts; IB4 staining was positive peritubular capillaries (Fig 2B-b) as well as ureteral capillaries (Fig 2B-c)

2. Heterotopic heart transplantation

a. Rat hearts-to-CTSs (Group 1)—After re-vascularization, rat heart grafts in CTS turned dark and were fully rejected in 35 minutes (median 22 minutes, Fig. 3A and 3B).

Histologically, interstitial hemorrhage and acute thrombi developed as well as neutrophil infiltrate in the heart grafts (Fig. 3C-a and C-b) while no obvious lymphocyte infiltrates were seen in the rejected graft. In order to determine if antibody deposition and complement activation were involved, frozen samples were stained for IgM, IgG, and C3c. IgM (Fig. 3D-a), IgG (Fig. 3D-b), and C3c (Fig. 3D-c) depositions were detected on small vessels, suggesting the antibody-mediated graft rejection. The deposition of IgM, IgG, and C3c were found on small vessel endothelial cells with interstitial hemorrhage, indicating that hyperacute rejection was caused by non-Gal-nAb mediated activation of the complement system.

b. CTS-to-Rat (Groups 2 and 3)—We performed 10 CTS-to-Rat heterotopic heart transplantation. Among these 10 animals, 7 received no immunosuppression (Group 2) while 3 recipients were treated intraperitoneally with FK506 at 1.0 mg/kg/day and leflunomide at 5.0 mg/kg/day (Group 3). Grafts in Group 2 were rejected in 4 days average (maximum survival 5 days), while 2 of 3 grafts in Group 3 continued to beat up to day 8 (average survival 8 days) (Fig. 4A). The third animal in Group 3 was intentionally euthanized on day 4 to compare with the histologic results of Group 2. Although grafts in Group 2 were rejected on day 4 on average, these grafts were not hemorrhagic but some petechiae were observed (Fig 4B)

Histologically, H&E staining showed acute cellular and antibody mediated rejection in Group 2. In addition to thrombi in vessels and interstitial hemorrhage, myocyte necrosis as well as focal lymphocyte infiltrates were observed (Fig. 4C-1 and 4C-2). In contrast to Group 2, the graft excised at day 4 in Group 3 showed no cellular infiltrate or hemorrhagic changes in the graft, indicating no histological evidence of T cell-mediated and/or antibody-mediated acute rejection (Fig. 4D). Grafts in Group 3 were eventually rejected at day 8 with mononuclear cell infiltrates (Fig. 4E). Infiltration of mononuclear cells in the intersitium and endoarteritis with thrombus formation were evident in the grafts, suggesting the development of T cell-mediated and antibody-mediated acute rejection by day 8.

Discussion

Recent advanced techniques of gene manipulation with better understanding immunologic barriers across xenogeneic barriers markedly improved graft survival of pig organs in non-

human primate (NHP) models from days to several months in the past 15 years (19–23). The results of GalT-KO pig organs into NHP confirmed that hyperacute rejection induced by incompatibility of 1,3 α Gal between pigs and primates is no longer a major hurdle in xenotransplantation. However this success also revealed that additional immunologic barriers have to be overcome before xenotransplantation can become a clinical reality. One of these hurdles is the presence of “non-Gal” antigens in pig tissues/cells that can trigger a reaction of the primate immune system. Antibodies to these antigens are referred to as anti-non-Gal Abs and mediate the acute and chronic rejection against xenografts (20, 24–27). To date, studies addressing the problem of anti-non-Gal Abs in xenotransplantation are still limited. Studies using pig cells obtained from various organs from WT as well as from GalT-KO pigs indicate that there are human anti-non-Gal Abs with the capacity to activate the complement and cause cell damage *in vitro* (8, 20, 28, 29) as well as ADCC-mediated cell damage (29). However, in most cases, the potential antigen(s) identified by these antibodies as well as the significance of these antibodies for graft damage *in vivo* are not known. Even though GalT-KO pig-to-NHP models could be used as *in vivo* studies for anti-non-Gal Abs (20, 24–26), some non-Gal antigens such as N-glycolylneuraminic acid (NeuGc) epitopes, the so called Hanganutziu-Deicher (HD) antigens, which are present in non-human primates but not in humans, cannot be studied in standard pig-to-NHP models (30, 31). In addition, there are obvious logistical problems in terms of expense, housing facilities, size and gestation times of NHPs which make difficult to characterize anti-non-Gal Abs and test treatment protocols in large animal models.

A number of small animal models such as mice, rats, guinea pigs and hamsters have been used as research models in xenotransplantation, however there are still many discrepancies between the pig and the primate physiology and immunology. Furthermore, small animal models using GalT-KO mouse elicit a weak humoral reaction by anti-non-Gal Abs and thus may not be ideal to clarify the underlying mechanisms of rejection (32–34). Therefore, in order to better understand mechanisms of non-Gal nAb-mediated rejection, a simple small animal model that is phylogenetically similar to primates would be extremely valuable.

CTSs are similar to rats in size, they are relatively inexpensive and do not pose the ethical challenges of large animal models. A recent whole genome sequencing study confirmed that the CTS has a closer affinity to NHP than rodents, indicating that they may be more suitable to study the pathogenesis of human diseases (13). CTS have been successfully employed as model animals for psychosocial stress (35), H1N1 virus (36), and hepatitis virus (37). The present study showed that CTSs have strong anti-non-Gal Abs. However, in contrast to humans and NHPs, we found that they have no anti-Gal Abs because they express α Gal antigens in their organs; this allows us to study non Gal nAb rejection as well as other immunologic barriers without the need to use GalT-KO swine. In addition, the combination of CTS and rat is an extremely valuable unique small animal model. A rat-to-CTS model can be used to determine mechanisms of non-Gal nAb and develop strategies to avoid humoral rejection; in the opposite direction a CTS-to-rat can be used as a concordant xenotransplant model. Although further studies including identification of the characteristics of nAb need to be performed, we conclude that CTSs are valuable small animals to study mechanisms and strategies to avoid non-Gal nAb mediated xenograft rejection.

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Abbreviations

BSA	bovine serum albumin
CTS	Chinese tree shrew
FK506	Tacrolimus
GalT-KO	α -1,3-galactosyltransferase knockout
IB4 lectin	α Gal specific lectin (GSIB-4)
LEF	Leflunomide
MGH	Massachusetts General Hospital
MHC	Major histocompatibility complex
nAb	Natural antibody
NHP	non-human primate
POD	Post-operative day
WT	wild type

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Figure 1. Determination of anti-Gal antibody and anti-non Gal antibody in sera of CTS
 (A). Anti-Gal IgM in serum of 5 baboons from MGH and 4 CTSs was assessed by BSA ELISA assays, Control group were using same volume of medium. Data are presented as mean $\pm$ SEM of more than three independent experiments. *** $P < 0.001$ vs. CTS group. (B) Anti-Non Gal antibody were clearly present in naïve CTS sera (anti-human IgM FITC (left) or IgG FITC (right)) assessed by flow cytometry. The numbers are only for the representative data of three separate experiments.

Figure 2. Gal antigen expression in hearts and kidneys

IB4 staining to assess Gal expression of heart (A) and kidneys (B). IB4 was positive in pig hearts (A-a), rat hearts (A-c) and CTS hearts (A-d), but was negative in GalT KO pig hearts (A-b). IB4 positive in rat kidneys (B-a), CTS kidneys (B-b) and CTS ureters (B-c). Data are representative of 3 independent experiments that showed similar results.

Figure 3. Rat-to-CTS heart transplantation (group 1) demonstrating hyperacute rejection
Heart graft survival (A) and representative gross findings of a rejected heart in CTS at 35 minutes. (B) Graft survival was calculated by the Kaplan-Meier method and compared by a log-rank test. Histology result of a hyperacutely rejected rat heart in CTS: HE staining (C-a $\times 200$, C-b $\times 600$), and immunofluorescence staining with anti-human antibody IgM (D-a), IgG (D-b) and C3 (D-c). Data are representative of 3 independent experiments that showed similar results.

Figure 4. CTS-to-Rat heart transplantation demonstrating acute rejection (groups 2 and 3)
 Heart graft survivals (A) and representative gross findings of a CTS heart in a rat without immunosuppression (B). Graft survival (A) includes results of group 1 (rejected in 35 min) to compare to the results of groups 2 and 3. Graft survival was calculated by the Kaplan-Meier method and compared by a log-rank test. Histology of CTS hearts in rats at day 4 without immunosuppression (group 2, C-a $\times 400$, C-b $\times 600$) and with FK506 (1.0 mg/kg/day) and leflunomide (5.0 mg/kg/day) IP daily that was intentionally euthanized on day 4 to provide a comparison with animals on group 2 euthanized at the same time point (group 3, D). In group 2, antibody-mediated acute rejection with lymphocyte infiltration developed in the graft by day 4. However, in group 3, no T cell-mediated and antibody-mediated acute rejection was evident in the grafts by day 4. Panel E demonstrates representative findings of rejected CTS heart grafts in group 3 (day 8). At day 8 in group 3, interstitial cellular

infiltrates and thrombi formation with endoarteritis were noted in the grafts, indicating the development of T cell-mediated and antibody-mediated acute rejection.

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