ORIGINAL RESEARCH

FK506 treatment in a Long-term chronic rejection rat model of small bowel transplantation

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Abstract

Background: Although acute rejection (AR) can be significantly improved by effective immunosuppressants, such as FK506, chronic rejection (CR) remains a major hurdle to long-term allograft survival after small bowel transplantation, in part because the pathogenic mechanisms of CR are, as yet, unknown. The rat orthotopic small bowel transplantation (OSBT) model has been used by a few researchers, but without long-term survival.

Methods: Rats were randomly divided into five groups: Group 1 (n=20), sham-operation rats; Group 2 (n=20), Lewis to Lewis; Group 3 (n=20), F344 to Lewis treated with FK506 (0.3 mg/kg/day); Group 4 (n=20), F344 to Lewis with FK506 (0.5 mg/kg/day); Group 5 (n=20), F344 to Lewis with FK506 (1.0 mg/kg/day). FK506 was administered intramuscularly to recipients on postoperative days (POD) 0-13, 20 and 27. Body weight, survival rate and histology were measured.

Results: Histopathological analysis revealed distinctive abnormalities of the allograft for all animals, including changes in villous architecture, interstitial fibrosis and intimal thickening; however, survival times were significantly increased with higher doses of FK506. Rats in Group 3 and Group 4 (low and moderate FK506 doses) survived 16-18 weeks, while recipients in Group 5 (high dose) survived 24-27 weeks.

Conclusion: FK506 treatment (1.0 mg/kg/day, intramuscularly administered to recipients on POD0-13, 20 and 27) can be used effectively to establish a rat OBST model of CR that will be useful for the study of the pathogenesis of CR and the effectiveness of various drugs.

List of Abbreviations

OSBT	orthotopic small bowel transplantation
HSBT	heterotopic small bowel transplantation
CR	chronic rejection
AR	acute rejection
POD	postoperative days
H&E	hematoxylin and eosin

Small bowel transplantation is indicated in patients with chronic, irreversible intestinal failure associated with failure or severe complications of total parenteral nutrition.¹ Despite recent advances in immunosuppressive strategies, surgical and perioperative techniques, and increased understanding of acute rejection of vascularized organs, the long-term survival of vascularized allografts has not improved, largely due to chronic rejection (CR). CR remains a major concern -

an indolent but progressive form of graft injury that eventually results in graft failure.^{2,3} It has been reported that ischemia reperfusion injury, alloantigen-dependent immune response and chronic delayed-type hypersensitivity may contribute to the development of CR. Additionally, many inflammatory cytokines are might also associated with the processes of CR.⁴

Development of a small bowel transplant model of CR would provide a means for studying immunosuppressive and tolerance-inducing protocols to prevent CR of these highly immunogenic allografts. Several experimental models have been established to study CR in small bowel transplants. Most models use rat heterotopic small bowel transplantation (HBST), and the relative low surgical damage and mortality are major advantages of this model. Compared with HSBT, orthotopic small bowel transplantation (OSBT) is more similar to the clinical situation; however, OSBT has been performed by only a few researchers and without long-term survival.⁵⁻⁹ Thus, the aim of this study was to establish a rat model in which chronic graft rejection of the orthotopic entire small intestine graft would develop in a long term and reproducible manner.

Materials and Methods

Animals

Inbred adult male F344 and Lewis rats, weighing 220-300 g, were obtained from Vitalriver Company, Beijing, China. The donors were 30-70 g smaller than the recipients. All rats were housed individually in standard animal facilities, maintained on 12-h light/dark cycles, and fed with rat chow and tap water *ad libitum* for one week before testing. Food was withheld from both donor and recipient animals for 24 h prior to surgery. The protocol was approved by the Animal Research Committee of the Nanjing University. All procedures were carried out in accordance with 'Principles of laboratory animal care' (NIH publication NO. 85-23, revised 1985).

Animals were divided into five groups: Group 1 (n=20) consisted of Lewis rats with sham-operations; Group 2 (n=20) isogenic group Lewis to Lewis; Group 3 (n=20) allogenic group F344 to Lewis treated with FK506 at a dose of 0.3 mg/kg/day; Group 4 (n=20) F344 to Lewis treated with FK506 at a dose of 0.5 mg/kg/day; and, Group 5 (n=20) F344 to Lewis treated with FK506 at a dose of 1.0 mg/kg/day. FK506 was intramuscularly administrated to recipients on POD0-13, 20 and 27.

Surgical procedures

OSBT was performed using standard microvascular techniques.⁸ Donor and recipient surgeries were performed under anesthesia induced with 100 mg/kg ketamine, intraperitoneally. The entire small bowel from the ligament of Trietz to 1cm from the ileocecal valve was removed on a vascular pedicle consisting of the superior mesenteric artery, a portion of the inferior abdominal aorta, and the portal vein cut distal to the splenic vein, which was previously ligated. The intestinal lumen was then flushed with saline to remove debris, and the vascular bed was flushed with cold 4°C heparinized saline.

The recipient was anesthetized in a similar fashion. After mobilization of the inferior vena cava and the aorta from surrounding connective tissue, transplantation was performed by anastomosing the graft superior mesenteric artery to the recipient infrarenal aorta and the graft portal vein to the recipient inferior vena cava in an end-to-side fashion using a running 9-0 prolene suture. After the graft blood supply was recovered, gauze sponges were used to press the anastomotic site. The recipient's own small bowel with its mesentery was resected from the ligament of Treitz to the ileocecal valve and replaced with the graft by performing two primary end-to-end small bowel anastomosis. The last gauze sponge was removed before closing the abdominal wall.

FIGURE 1. Survival curve of rats in three FK506 treatment groups: all the recipient rats in Group 5 (high dose, 1.0 mg/kg/day) survived 24 -27 weeks; all rats in Group 4 (moderate dose, 0.5 mg/kg/day) survived 16-18 weeks and all rats in Group 3 (low dose, 0.3 mg/kg/day) survived 16-17 weeks.

Statistical Analysis

Statistical analysis of the differences in body weight increase during allogenic groups was performed using Student's t test, values were considered significant at $p < 0.05$. Kaplan-Meier method and the long-rank test were performed by SPSS software (SPSS Inc. Chicago, IL) for animal survival and body weight increase.

Results

Survival Curve

After OSBT, recipients in allogenic groups developed CR-associated symptoms of diarrhea and severe weight loss, which eventually led to death. All the animals in the Group 2 and Group 5 survived up to POD180, and all recipients in Group 5 died before 27 weeks. Most rats in Group 3 and Group 4 survived up to POD120 but all died before 18 weeks (Figure 1).

Summary of postoperative mean body weight changes

Recipients undergoing transplantation lost weight during first week post-transplant, then started to a slow recovery and increase in body weight. In three allogenic groups, Group 5 showed the maximal body weight increase while the minimal increase on body weight appeared in Group 3 ($p=0.007$). (Figure 2).

Histology

All of the allografts demonstrated one or more histological features of CR. Histopathological analysis revealed distinctive abnormalities of the allograft: changes of villous architecture, interstitial fibrosis, leukocyte infiltration and intimal thickening. All the allografts displayed intimal thickness in some degree. Most of the arteries in Group 3 manifested numerous inflammatory cell infiltration, severe intimal hyperplasia and were nearly total obliteration, which was consistent with severe features of CR. Arteries in Group 4 showed numerous inflammatory cell infiltration

Postoperative care and observation

Rats were kept on a warming blanket and put under a heating lamp until recovery from anesthesia. All animals had access to pellets and water. Death within five days due to arterial anastomosis leakage or thrombus was considered as a technical failure and excluded. The animal's general state of health (appearance, posture, feeding, activity and body weight) was assessed daily. Allografts and isografts were removed at the time of clinical rejection (body weight reduced > 20 g/d) or, if continuing to appear normal, at close to 180 days after transplantation for histological analysis.

Histological Analysis

Once sacrificed, mesentery grafts were harvested, after flushing the intestinal lumen with normal saline, tissues were fixed in 10% formalin at 4 °C for 24 hours, dehydrated in alcohol, and cleared in xylene before being embedded in paraffin. Sections (5 μ m) were dewaxed in xylene, hydrated, and stained with hematoxylin and eosin.

FIGURE 2. The mean body weights of different experimental groups are shown. The increase of body weight in syngeneic group was similar to that seen for the sham-operation group. In three allogeneic groups, Group 5 showed the maximal body weight increase while the minimal increase on body weight appeared in Group 3.

tion, but only mild intimal hyperplasia and narrow lumen. Many arteries in Group 5 displayed only mild to moderate intimal hyperplasia (Figure 3).

Discussion

CR remains the major obstacle to long-term allograft survival after clinical intestinal transplantation; however, the precise pathogenetic mechanisms leading to CR are largely unknown. The development of representative and reproducible animal models of OSBT will play a critical role in improving allografts long-term survival.¹⁰

Rat is the most frequently used animal model for SBT because this experimental model is inexpensive and ethically acceptable, and various inbred strains with well-defined histocompatibility properties are available. F344 and Lewis rat strains were used in this work because the strains differ at the RT1.C region, a second class I locus of the rat MHC, and at non-MHC regions (e.g., RT6). Products of the RT1.C class I MHC locus are associated with chronic rejection of

FIGURE 3. Pathological changes of allografts (hematoxylin and eosin, x400). (A) Mesenteric artery showed numerous inflammatory cell infiltration, severe intimal hyperplasia and with near total obliteration; (B) Mesenteric artery displayed numerous inflammatory cell infiltration, mild intimal hyperplasia and narrow lumen; (C) Mesenteric artery showed only mild intimal hyperplasia.

TABLE 1. Previously published survival times for OSBT recipient rats treated with FK506

OSBT	FK506 dose and treatment protocol	survival time (days)
Wesserberg N ⁶	1.0 mg/kg/day (im) POD0-5	60
Meyer D ⁷	2.0 mg/kg/day (im) POD0-5	100
Murase N ⁸	1.0 mg/kg/day (im) POD0-13,20,27	>150
Guo WH ⁹	2.0 mg/kg/day (im) preoperative 3 days +0.3 mg/kg/day (im) POD0-14	86.4±14.1

OSBT: orthotopic small bowel transplantation; POD: postoperative days; im: intramuscular injection

vascularized allografts.. This F344 to Lewis SB heterotopic transplant model is a useful model that reproduces significant features of CR; however, most studies used the HSBT model due to significantly lower mortality than that found with the OSBT model. Orloff *et al.*¹¹ treated graft recipients with a short-term course of low-dose Cyclosporine A. Wente and co-workers used FK506 in rat OBST¹² in order to better represent the physiologic state of small intestinal function and to overcome the severe toxicity of Cyclosporine A. The mean survival time was found to be three months with various FK506 treatments (Table 1).⁶⁻⁹ Other immunosuppressants or combination of drugs were tried but the results were contradictory.¹³ Murase *et al.*⁸ performed OSBT from Brown Norway to Lewis rats underwent a short course of FK506 treatment (1mg/kg/day, POD0-13, 20, 27): all recipients survived over 150 days but CR was severe in the intestine. The BN-to-Lewis rat strain combination is strongly histoincompatible which led to severe rejection, while F344-to-LEW rat inbred strain combination showed less histoincompatible, so the doses of FK506 in our study should be lower. Consequently, doses of FK506 ranged from high (1.0 mg/kg/day) to median dose (0.5 mg/kg/day) to low (0.3 mg/kg/day). FK506 was administrated intramuscularly to recipients on POD0-13, 20 and 27. Without immunosuppressants, most recipients died of AR, so a control allogenic group without FK506 was not used in the study.

According to the survival curve, FK506 treatment prolongs the survival times of all allogenic rats up to 4 months. Of the three allogenic groups, the high dose

group (Group 5) had the longest survival times (over 6 months) and the most rapid post-surgical weight gains. Histological analysis showed that all allografts displayed one or more features of CR and most showed intimal thickness in some degree; from mild intimal hyperplasia to total occlusion but that CR features were less pronounced with higher doses of FK506. It appears that FK506 treatment improved recipients (F344 to Lewis) survival, and that the highest dose of FK506 (1.0 mg/kg/day, intramuscularly administrated to recipients on POD0-13, 20 and 27) was the most suitable to establish a long-term rat OSBT model of CR.

With this long-term survival model, researchers can now study and understand the various mechanisms of CR, and evaluate the ability of immunosuppressants or other drugs to ameliorate the damage caused by CR. Ma *et al.*¹⁴ demonstrated that fish oil has an immunosuppressive effect on rat allogenic small bowel transplantation and might ameliorate the arteriosclerosis of the grafts.

Conclusions

We demonstrated that the immunosuppressive protocol (FK506 1.0mg/kg/day, intramuscularly administrated to recipients on POD0-13, 20 and 27) effectively prolongs the OSBT recipient rats survival by up to half a year. This model will be useful for the detailed study of the mechanisms of CR and the effectiveness of various drugs to prevent or delay the development of CR. Ensuring that significant nephrotoxicity does not occur with this approach will also be an important component of future studies.

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