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Chapter

Controllable Immunosuppression in Pigs as a Basis for Preclinical Studies on Human Cell Therapy

Shin Enosawa and Eiji Kobayashi

Abstract

Along with a growing interest in regenerative medicine, pigs are becoming a popular model for preclinical studies on human cell therapy. Due to pharmaceutical species difference and inability to self-medicate, specific modification and care are necessary in immunosuppressive regimen for pigs. Here, we summarize recent literature on immunosuppression in pigs for experimental transplantation. Based on literature and our own experiences, a practical protocol has been proposed in this report. In early studies of allogeneic organ transplantation, recipient pigs were administered cyclosporine or tacrolimus, and mycophenolate mofetil at slightly higher dose than that in human cases, because of relatively poor effectiveness of the drugs in pigs. Steroids may be effective but sometimes can cause debilitating side effects. Cell transplantation studies follow the basic protocol, but it remains to be clarified whether the smaller graft mass, even if it is xenogeneic, requires the same scale of immunosuppression as organ transplantation. To obtain reliable results, the use of gastrostomy tube and blood trough level monitoring are highly recommended. Nonpharmaceutical immunosuppression such as thymic intervention and the use of severe combined immunodeficient pigs have also been discussed.

Keywords: pig, experimental transplantation, immunosuppression, human cell therapy, regenerative medicine

1. Introduction

The number of preclinical studies conducted using pigs has been increasing, especially in the field of cell therapy [1]. The merits of using pigs include (1) size advantages that enable to mimic clinical procedures; (2) availability of various experimental pigs such as miniature, microminiature, and gene-engineered strains; and (3) worldwide trend of discouragement of using dogs as research models.

However, immunosuppressive treatment has not been established well in pigs. Initially, pigs were used as models for performing allogeneic organ transplantation; now, the hope is to use them as xenogeneic organ donors. In the latter case, immunosuppressive protocols have been designed for primate recipients, while the number of reports mentioning immunosuppressive protocols for xenogeneic transplantation in pigs is unexpectedly few. In addition, insufficient medications are occasionally found in studies conducted by researchers who are not accustomed to organ transplantation.

We summarize here the pharmaceutical immunosuppressive regimens in experimental transplantation of organs, tissues, and cells in pigs (**Table 1**), as well as highlight the usefulness of thymic intervention and severe combined immunodeficient (SCID) pigs. Finally, we propose an appropriate introductory protocol that will fit human cell and tissue transplantation.

Study design	Pigs	Immunosuppression
In vitro culture, IC_{50} estimation [2]	Unknown	Cys, Tac, Aza, Rap, MMF, MP
Orthotopic allogeneic small bowel transplantation [3, 4]	Large White × Landrace, 26.4 ± 4.7 kg	Tac 0.43 ± 0.14 mg/kg/day, po (keep trough 5–15 ng/ml), MMF 10 mg/kg × 2/day, po
Trough level determination [5]	Yorkshire × Landrace, 22–30 kg	Tac 0.25 mg/kg × 2/day, po, MMF 20 mg/kg × 2/day, po
Orthotopic allogeneic forelimb transplantation [6, 7]	Outbred farm pig, 13–24 kg (6–8-week-old)	Cys 40 mg/kg/day, po (keep trough 100–300 ng/ml) or Tac 1.5 mg/kg/day, po (keep trough 4–8 ng/ml), MMF 500 mg/day, MP and P (tapered)
Orthotopic allogeneic (Class I disparate) kidney transplantation [8–10]	MHC-defined miniature swine, 5–7-month-old	Cys 10–13 mg/kg/day, iv (keep trough 400–800 ng/ml) [8, 9] or Tac 0.15 or 0.30 mg/kg/day, iv (keep trough 20–40 or 45–80 ng/ml) [10]
Heterotopic allogeneic (Class I disparate) heart transplantation [11]	MHC-defined miniature swine, 3–6-month-old	MMF 1.5 g × 2 /day, iv or Cys 10–13 mg/kg/day, iv (keep trough 400–800 ng/ml)
Immortalized human hepatocytes transplantation to liver [13]	Landrace, 6 kg	Tac 0.5 mg/kg/day, im
Human bone marrow mesenchymal stem cell transplantation to myocardium [14]	Domestic pig, 35–40 kg	Cys 5 mg/kg/day, route unknown
Human umbilical mesenchymal stem cell transplantation to cardiac muscle [15, 16]	Yorkshire, size and age unknown	Cys 4–10 mg/kg/day, po, 1–2/day, some cases treated with steroids
Human iPS cell-derived cardiomyocyte sheet to endocardium [18]	Minipig, 20–25 kg	Tac 0.75 mg/kg/day, MMF 500 mg/day, Pl 20 mg/day
Human ES cell-derived retinal sheet to retina [19]	Yucatan minipig	Cys, po, details unknown
Orthotopic allogeneic (Class I disparate) kidney transplantation [20]	MHC-defined miniature swine, 3–6-month-old	Thymectomy and donor thymus transplantation
Human hepatocyte transplantation to spleen [21]	Microminiature pig, 13–16-month-old	Neonatal thymectomy
Human artificial vascular tubes for neck arteriovenous shunt [22]	Göttingen minipigs, 6–7-month-old, ≥15 kg	Thymectomy and splenectomy, and Tac 0.5 mg/kg/day, MMF 60 mg/kg/day, prednisolone 20 mg/day, po

Abbreviations: half maximal inhibitory concentration (IC_{50}); major histocompatibility complex (MHC); cyclosporine (Cys); tacrolimus (Tac); azathioprine (Aza); rapamycin (rap); mycophenolate mofetil (MMF); methylprednisolone (MP); prednisone (P); prednisolone (Pl); per os (po); intravenous injection (iv); intramuscular injection (im).

Table 1.
Summary of immunosuppressive protocols.

2. Role of species differences in the effectiveness of immunosuppressants in vitro

The half maximal inhibitory concentration (IC_{50}) of major immunosuppressants was reported to be higher in mitogen response of pig lymphocytes than that in human lymphocytes [2]. The IC_{50} of cyclosporine, tacrolimus, and azathioprine was 19.1 times [1.72 μg/ml (pig) vs. 0.09 μg/ml (human)], 13.0 times [2.99 ng/ml (pig) vs. 0.23 ng/ml (human)], and 11.0 times [1.43 μg/ml [pig] vs. 0.13 μg/ml (human)] higher in pigs than in human lymphocytes, respectively. The species differences decreased in case of rapamycin and mycophenolate mofetil (MMF); IC_{50} values of rapamycin and MMF were 2.28 times [2.05 ng/ml (pig) vs. 0.90 ng/ml (human)] and 1.45 times [10.75 ng/ml (pig) vs. 7.42 ng/ml (human)] higher in pigs than in humans, respectively. In contrast, the IC_{50} value of methylprednisolone in pigs was only 0.41 times [0.11 μg/ml (pig) vs. 0.27 μg/ml (human)] the value in humans. These results suggest that the blood concentration of calcineurin inhibitors should be kept higher in pigs than in humans to suppress blast formation of lymphocytes in transplantation studies.

3. Immunosuppressive medications in allogeneic transplantation

In allogeneic orthotopic small bowel transplantation, high-dose tacrolimus monotherapy and low-dose tacrolimus-MMF combination therapy were compared using Large White-Landrace pig strain as donors and recipients (both weighing approximately 26 kg) [3, 4]. Tacrolimus dose was controlled to keep trough at 15–25 ng/ml (high-dose group) or 5–15 ng/ml (low-dose group). The average dosage amount of tacrolimus in the high single dose group was 0.3 mg/kg/day intramuscularly from the day of operation to day 6 and 0.61 ± 0.26 mg/kg/day via gastrostomy after day 7. In the low-dose combination group, recipients were administered 0.1 mg/kg of tacrolimus intramuscularly on the day of operation and 0.43 ± 0.14 mg/kg/day (average) of tacrolimus and 10 mg/kg twice a day of MMF via gastrostomy. All recipients in the high single dose group died within 46 days, while 7 out of the 10 recipients in the low-dose combination group survived for more than 60 days; the nontreated controls died within 15 days [3]. The subgroup study of tacrolimus-MMF combined group revealed that the recipients with low trough level of tacrolimus showed better survival, suggesting that higher trough level increases side effects of infection [4]. In general, the protocol consisting of calcineurin inhibitors (cyclosporine or tacrolimus) and MMF suppresses the immune responses of T and B cells, respectively, and the combination treatment leads to effective immunosuppression with low side effects.

A pharmacokinetic study recommended the oral administration of 0.25 mg/kg of tacrolimus and 500 mg of MMF at 12 h intervals to pigs weighing 22–30 kg [5]. MMF dose was calculated to be around 20 mg/kg at each administration. The trough level of tacrolimus was kept at 5–15 ng/ml.

When orthotopic forelimb transplantation was performed between outbred pigs weighing 13–24 kg, recipients were administered cyclosporine or tacrolimus and MMF orally once a day [6, 7]. The desired trough levels were 100–300 ng/ml in case of cyclosporine and 3–8 ng/ml for tacrolimus. A total of 500 mg of MMF was administered, i.e., 21–38 mg/kg. They also used steroids; 500 mg of methylprednisolone was injected intravenously during the operation, and 2.0 mg/kg/day of prednisone was given on the first postoperative day and then tapered by 0.5 mg/kg/day every 3 days to a maintenance dose of 0.1 mg/kg/day until the end of observation period (90 days) [6] or for the first 30 days [7].

The Massachusetts General Hospital group uses genetically defined mini pigs, swine leukocyte antigen (SLA)^{gg} (class I^{c}/class II^{d}) donors, and SLA^{dd} (class I^{d}/class II^{d}) recipients [8–11]. In orthotopic kidney transplantation, 10–13 mg/kg of cyclosporine once a day, administered with a catheter to the external jugular vein, kept the trough level at 400–800 ng/ml [8, 9]. The first 12-day administration resulted in the survival of well-functioning major histocompatibility complex (MHC) class I-disparate kidney grafts for over 90 days regardless of use of steroids. In another study [10], continuous intravenous injection of 0.15 or 0.30 mg/kg/day of tacrolimus treatment kept the drug level at 20–40 or 45–80 ng/ml, respectively, and the first 12-day administration resulted in well-functioning kidney grafts that survived for over 5 months. In addition, the high-dose regimen achieved successful engraftment of MHC class I^{c}/class II^{c}-mismatched kidney. The same group also compared the separate effect of cyclosporine and MMF on the survival of class I-disparate heterotopic heart graft [11]. The treatment protocol of cyclosporine was the same as above [8] and MMF was administered at 1.5 g twice a day through a catheter into the external jugular vein to keep the trough level at 3–5 μg/ml. The survival days of the test heart grafts were 53 ± 7.5 days (mean ± SD) and over 124 days in cyclosporine and MMF groups, respectively. The graft vascular changes were also mild in the MMF group.

4. Immunosuppressive medications in xenogeneic transplantation

Because pigs are hoped to be a xenogeneic donor, the major objectives in pig experiments include establishing xenogeneic antigen-free pigs and developing strategies for long-term survival in nonhuman primates [12]. In such studies, immunosuppression is almost equivalent to human clinics using tacrolimus, MMF, and antibody remedies. Pig recipients in xenotransplantation appear in the preclinical studies of human cell and tissue therapy.

In a short-term experiment of intrahepatic transplantation of human hepatocyte cell line, 0.5 mg/kg of tacrolimus was injected intramuscularly for 7 days [13]. When pigs received xenogeneic human-lined hepatocytes, the recipients survived D-galactosamine-induced hepatic injury. Human albumin appeared in the recipient serum 2 days after transplantation but disappeared at day 7, suggesting that the cells survived only for a few days.

Intramyocardial transplantation of mesenchymal stem cells had been actively investigated in not only basic research but also clinical practice. Because of the size advantage, pigs were used for preclinical studies to explore proof of concept by mimicking clinical procedure. Human bone marrow-derived mesenchymal stem cells labeled with radioactive indium (^{111}In) were transplanted in porcine myocardium via catheter inserted from a femoral artery and traced by whole body scanning for 6 days [14]. Recipient pigs were orally treated with 5 mg/kg of cyclosporine from 3 days before to 6 days after cell transplantation. Immunosuppressed pigs retained the radioactivity far longer than nontreated controls. In another study, pigs received human umbilical mesenchymal stem cells in artificial cardiac infarct area and were administered 5 mg/kg of cyclosporine orally twice a day, from the day before to 8 weeks after cell transplantation [15]. In a similar study, 10 mg/kg of cyclosporine was administered orally, twice a day from 3 days before to 8 weeks after the cell transplantation [16]. As a more sophisticated approach, cardiomyocyte sheet transplantation is being undertaken [17, 18]. When cell sheets consisting of human induced pluripotent stem (iPS) cell-derived myocytes were transplanted onto the epicardium of minipigs (weighing 20–25 kg) mimicking clinical trial, 0.75 mg/kg/day of tacrolimus, 500 mg/day of MMF, and 20 mg/day of prednisolone were administered from 5 days before to 8 weeks after the transplantation [18].

Transplantation of human embryonic stem cell-derived retinal pigmented epithelium was also performed in pigs [19]. Although immunosuppression was precarious, cyclosporine was added in the feed and their blood level 2 h after administration was only 1 pg/ml, and the graft tissue was detectable after 3 months. Because retina is known as an immune-privileged site, additional immunosuppression may not be necessary.

5. Nonpharmaceutical immunosuppression: thymic intervention and SCID pigs

Studies on genetically defined minipigs by Massachusetts General Hospital group emphasize the role of thymus in the establishment and maintenance of immunological tolerance. As quoted above [8], the 12-day administration of cyclosporine induced the long-term engraftment of MHC class I-disparate kidneys, but not if the thymus was removed. They also stated that old recipients tend to be difficult to establish tolerance [9]. These observations lead to the concept of tolerance induction by thymic transplantation. In this, unlike conventional thymic tissue transplantation, the donor thymus is transplanted by vascular anastomosis that assures immediate and perfect function of the thymus. Three weeks after the complete removal of thymus, the recipient was transplanted with MHC fully-disparate donor thymus in the neck region and infused continuously with 0.15 mg/kg/day of tacrolimus (trough level, 30–40 ng/ml) for 12 days. After 3–4 months, the recipient accepted a kidney from the thymus donor without immunosuppression [20].

Previously, we reported the effectiveness of thymectomy on the acceptance of xenogeneic human hepatocytes and artificial vascular tubes [21, 22]. Upon hepatocyte transplantation, the blood human albumin levels were higher in neonatally-thymectomized microminiature pigs than in nonthymectomized controls [21]. In another study, thymus and spleen were removed from Göttingen minipigs aged 6–7 months (≥ 15 kg), followed by the administration of 0.5 mg/kg/day of tacrolimus, 60 mg/kg/day of MMF, and 20 mg/day of prednisolone [22]. Seven days after the removal, an artificial vascular tube made from human fibroblasts was transplanted in between a carotid artery and a jugular vein to form an arteriovenous shunt. While the shunt was obstructed completely by thrombus 2 weeks after the operation in pigs without the removal of thymus and spleen, the shunt was functional in pigs with thymectomy and splenectomy, even though the immunosuppressive treatment administered was equal.

Finally, we would like to refer briefly to the availability of SCID pigs in preclinical study on human cell therapy. According to a well-constructed review [23], there are 11 SCID pig strains so far; one was naturally found and others were genetically modified. The mutated genes in these strains are *ARTEMIS* (a gene encoding a nuclear protein that is involved in V(D)J recombination and DNA repair), *interleukin 2 receptor gamma chain (IL2RG)*, *recombination-activating genes (RAG)1*, and *RAG2*. Three strains have double mutations, namely *RAG1* and *2*, *RAG2* and *IL2RG*, and *ARTEMIS* and *IL2RG*. In accordance with gene function, each strain lacks specific immune-competent cell lineages such as T, B, and natural killer (NK) cells. Human cell transplant experiments were reported as iPS cell teratoma formation [24] and ovarian cancer engraftment [25], both of which did not focus on preclinical study on human cell therapy. SCID pigs need the highest antibacterial care because of their vulnerability to infection. Indeed, they have been reported to survive for only 6 months at the longest [23]. In addition, as general features of mutant pigs, there are diversities in phenotypic severity and small litter size. If

these difficulties can be overcome, SCID pigs will be useful experimental animals for preclinical study on human cell therapy.

6. Conclusion

Due to the lack of identifiable sign of rejection, immunosuppression in cell transplant experiment is hard to control. Successful protocol is established only based on case-by-case experiences. Here, we suggest an introductory regimen of immunosuppression in human cell and tissue transplantation into pigs using tacrolimus and MMF. Preliminary doses are 0.5 mg/kg of tacrolimus orally and 40 mg/kg of MMF orally, and the administration should start 3 and 5 days before transplantation, respectively. Drugs can be administered by mixing in the powdered feed before transplantation; however, after transplantation, it should be given through a gastrostomy tube to assure the dosage in order to not be affected by appetite. Periodical examination of drug trough levels is indispensable and should be reflected in subsequent dose. Steroids should be carefully tested because their immunosuppressive dose has a risk of side effects such as gastrointestinal ulcer and systemic over immunosuppression. In addition, unavailability of exogenous steroid monitoring makes dosage control difficult. If surgical skill is available, the combination of thymectomy and splenectomy is recommended. Since the graft mass of cell and tissue transplantation is far smaller and not fully vascularized than organs, the recipients may need less immunosuppression. Data accumulation and optimization are desired in this field.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Shin Enosawa* and Eiji Kobayashi
Department of Organ Fabrication, Keio School of Medicine, Tokyo, Japan

*Address all correspondence to: enosawa-s@ncchd.go.jp

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