



Porcine Xenotransplantation to Primates*

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ABSTRACT : Xenotransplantation is a hot topic currently, since the demand for diverse organs is increasing in patients. Among many species, pigs are suitable animals for xenotransplantation as they share many anatomical and physiological characteristics with humans. This review article provides an overview of porcine xenotransplantation and the rejection of pig xenotransplants in primates, and use of genetically modified and cloned pigs in xenotransplantation. It also highlights major target organs in porcine xenotransplantation and virus infection in xenotransplantation. (**Key Words :** Xenotransplantation, Pig, Rejection, Virus Infection)

INTRODUCTION

Transplantation is one of greatest challenging achievements and saves thousands of lives each year. It also improves the quality of patients who present each year with organ failure. However, the transplanted organs are very insufficiency owing to the overwhelming demands for donated organs. Xenotransplantation may be one of the best possible approaches to solving the severe shortage of human donors, which greatly limits progress in clinical transplantation. Therefore, xenotransplantation is currently a hot topic in bio-organ of biomedical research. Among many species, pigs are suitable animals due to easier animal husbandry, comparatively similar anatomical and physiological similarities to human organs (Hughes, 1986; Pereira-Sampaio et al, 2004; Vodicka et al, 2005). These

reports suggest that pigs can be used as alternative to the shortage of human organs, if we produce appropriate and optimal pig strain without immune response to human (Vodicka et al, 2005; Puga Yung et al, 2009). However, many crucial answers on efficacy and safety will ultimately only be solved by well designed and controlled solid organ xenotransplantation trials on humans. Therefore, further research on the potential effects of crossing the species barrier in pig-to-primate model is essential before clinical application is acceptable (Ravelingien et al., 2004; Ekser et al., 2009). This review article provides an overview of the rejection in pig xenotransplantation to primates, and genetically modified and cloned pig in xenotransplantation. It also highlights major target organs in porcine xenotransplantation and virus infection in xenotransplantation.

REJECTION IN PORCINE XENOTRANSPLANTATION

The most profound barrier to pig-to-primate xenotransplantation is the rejection of the grafted organ by a cascade of immune mechanisms commonly referred to as hyperacute rejection (HAR), acute vascular rejection (AVR)/humoral xenograft rejection (AHXR), immune cell-mediated rejection, and chronic rejection. HAR leads to graft rejection within minutes, was considered as first barrier to pig-to-human xenotransplantation. Gala1, 3Gal (Gal), a particular carbohydrate, is majorly implicated in the development of hyperacute rejection of pig organs transplanted into humans and non-human primates (Good et

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al., 1992; Cooper et al., 1993), since α Gal epitope is absent in humans, apes, and monkeys. Genetically engineered “knock-out” pigs that lack the α 1,3-galactosyltransferase gene (GalT-KO) and thus do not express the Gal oligosaccharide have been used to improve the hyperacute rejection (Hisashi et al., 2008; Shimizu et al., 2008). Therapeutics for Gal also has several problems, since blocking of Gal attenuated hyperacute rejection but not AVR injury (Pierson, 2009). Classical complement pathways also are involved in the hyperacute rejection. Inefficient coagulation inhibitory pathways involving endothelial proteins such as thrombomodulin and tissue factor pathway inhibitor (TFPI) result in increase of thrombosis, although porcine endothelium exposed to human blood have somewhat similar mechanism compared to that of human endothelium exposed to human blood (Pierson et al., 2009). AVR/AHXR is defined as a rejection that begins within 24 h after transplantation and gradually destroys the graft (Platt, 1998). AVR is generally known to be initiated by xeno-reactive antibodies, including non-Gal antibodies and subsequent activation of the graft endothelium, the complement and the coagulation systems. It occurs within days by the humoral and cellular action of B lymphocytes and T lymphocytes. Antibodies targeting the CD 154/CD40 pathway were used to improve the rejection of T cell and B cell response to xenoantigen (Kawai et al., 2000; Kirk and Harlan, 2001). Direct xenorecognition and indirect xenorecognition are available routes for presentation of pig antigens to primate T cells (Pierson et al., 2009). CD40 and the B7 family proteins (CD80, CD86, and others), which are expressed on “antigen presenting cells”, bind to CD154 and CD28 or CTLA-4, respectively, ligands which are expressed mainly on responding T cells. Therefore, blocking CD 28 or the F7 family can be a targeting for the rejection of porcine xenotransplantation into primates.

Several lines of evidence indicates that NK cells play an important role in rejection of porcine xenografts into human (Inverardi et al., 1994; Khalfoun et al., 2000). The initiating cause of failure of pig xenografts may be antibody-mediated injury to the endothelium, leading to the development of microvascular thrombosis. Therefore, potential contributing factors to the development of microvascular thrombosis are the presence of preformed anti-non-Gal antibodies, the development of very low levels of elicited antibodies to non-Gal antigens, natural killer cell or macrophage activity, and inherent coagulation dysregulation between pigs and primates (Ekser et al., 2009).

PORCINE GENETIC ENGINEERED MODEL FOR XENOTRANSPLANTATION

Techniques for porcine genetic modification are crucial

for xenotransplantation research. For germ-line gene transfer, retroviral vector (Cabot et al., 2001), lentiviral vectors (Hofmann et al., 2003; Whitelaw et al., 2004), sperm-mediated gene transfer (SMGT) (Lavitano et al., 2006) and somatic cell nuclear transfer (SCNT) were used. Lentiviral gene transfer has been used in a variety of experiments to transduce cells with various transgenes owing to high efficiency (Sachs and Galli, 2009). SMGT has also been used with a nonviral episomal vector (Manzini et al., 2006). SCNT has become the optimal tool for generating pig animals from genetically engineered somatic cells. Recently, the Cre site-specific DNA recombinase system, a powerful tool for manipulating DNA *in vivo*, has been used for porcine xenotransplantation (Prather et al., 2003; Lunney, 2007). A reporter pig strain containing the EGFP gene driven by the CMV promoter, was used, in which the EGFP gene is expressed only after Cre-mediated excision of loxP-flanked stop sequences. The EGFP will be conditionally expressed in the resulting embryos and adult pigs. The reporter pig strain is capable of generating recombinant animal tissue in which Cre-mediated excision events can be studied *in vivo* in a variety of experimental contexts (reviewed in Li et al. in 2009).

Engineered pigs are of great value for research and commercial applications and could serve as models for human disease (Brunetti et al., 2008). Porcine genetic engineered model is based upon the immune rejection abovementioned. Since HAR is mediated majorly by the antibody of Gala1, 3Gal (Gal), it is generally accepted that Gal KO is an important prophylactic genetic modification for xenotransplantation of vascularised organs. d'Apice and Cowan (2009) insisted that Gal KO pig is the basic “standard platform” pig on which other genetic modifications can be assembled. HAR may be overcome by using genetically modified donor pigs lacking functional GGTA1 expression. Competitive glycosylation strategies by transgenic expression of human α -1,2-fucosyltransferase (α -1,2-FT) or human β -1,4-N-acetylglucosaminyltransferase III (GnT-III) were used to reduce α -1,3-Gal epitopes on porcine cells (reviewed in Klymiuk et al. in 2010). Transgenic pigs for human complement-regulatory proteins (CRPs) such as human decay-accelerating factor (hDAF), CD46 and CD59 have been characterized (Byrne et al., 1997; Cozzi et al., 1997; Diamond et al., 2001; Lee et al., 2010). Transgenic pigs for natural killer cell-mediated rejection were also established (Weiss et al., 2009). Recently triple transgenic (CD59/DAF/hTM) pigs are used to in pig xenotransplantation (Petersen et al., 2009). Therefore, the use of transgene or knock-out pig organs in pig-to-non-human primate xenotransplantation can prevent HAR, AVR, and immune-mediated rejection, eventually extending

xenograft survival.

MAJOR TARGET ORGANS OF PORCINE XENOTRANSPLANTATION

The lack of sufficient numbers of donor organs resulted in the deaths of the patients. Xenotransplantation using pig organs could resolve the shortage of suitable donor organs (Cooper et al., 2002). The initiating cause of failure of pig xenografts may be antibody-mediated injury to the endothelium, leading to the development of microvascular thrombosis. In this paper, we would like to briefly introduce the major organs of porcine xenotransplantation.

Porcine Liver transplantation

The liver is the primary site of xenotransplantation. Liver transplantation offers several benefits for the treatment of patients with liver failure or acute liver failure and end-stage chronic liver disease. According to the review of Esker et al. (2009), during the past 13 years, 30,000 patients have died waiting for a liver transplantation. Ramirez et al. (2000) transplanted livers from wild-type pigs or pigs transgenic for the human complement-regulatory protein, human decay-accelerating factor (hDAF), into immunosuppressed baboons. Makowka et al. (1995) reported that there was an attempt to porcine liver xenotransplantation to human, eventually resulting in the death without success. Recently, Esker et al. (2010) reported that after the transplantation of genetically engineered pig livers into baboons i) many parameters of hepatic function, including coagulation, were normal or near normal; ii) there was evidence for production of pig proteins, including coagulation factors; and iii) these appeared to function adequately in baboons although interspecies compatibility of such proteins remains to be confirmed. This report gives us good possibility that the liver xenotransplantation may be applicable to clinical usage in human in future. Some reports suggest that pig livers may be rejected less vigorously than other pig organs (Tuso et al., 1993; Tector et al., 2001). They observed that xenoperfused pig livers may function for up to 5 h, in contrast to porcine kidneys or hearts. According to the reports of Ramirez et al. (2000; 2001), 3 baboons transplanted with livers from unmodified pigs survived for <12 h with the response of HAR but two baboons transplanted with livers from pigs transgenic for hDAF survived for 4 and 8 days. These reports suggest that if HAR is abrogated (by the presence of hDAF), the porcine liver can maintain reasonable levels of coagulation factors and protein in the baboon for up to 8 days (Hara et al., 2008). Porcine hepatocyte transplantation also could be proposed as a method to support patients with liver

insufficiency (Gewartowska and Olszewski, 2007).

Porcine cardiac xenotransplantation

Use of a pig heart as a bridge to allotransplantation could be a solution due to heart deficiency organ. Orthotopic pig-to-baboon heart transplantation is the accepted preclinical model for cardiac xenotransplantation in humans, although heterotopic thoracic pig-to-baboon heart transplantation was reported (Bauer et al., 2010). When the hearts from hdAF transgenic pigs were perfused with human blood, HAR was avoided and the hearts were relatively metabolically and functionally stable (Smolenski et al., 2007). Hisashi et al. (2008) observed that no hyperacute rejection developed and one graft survived up to 6 months after transplantation. However, they also indicated that all GalT-KO heart grafts underwent graft failure with AHXR and/or chronic rejection. In recent report, the role of alpha-1,3-galactosyltransferase (alpha-Gal) antigen in valve calcification by comparing alpha-Gal-positive and alpha-Gal-deficient (GT-KO) pig pericardium was examined to improve the use of heart valve in xenotransplantation (Lila et al., 2010). Qv et al. (2009) observed that complement is dysregulated in heart xenotransplantation in pig-to-primate models. These reports suggest that the rejection should be overcome in porcine heart xenotransplantation. Cooper et al. (2010) provided several guidances as preliminary requirements in animal models in porcine xenotransplantation. i) Heterotopically-placed pig heart grafts survive and function fairly consistently (eg, 7 of 10) for at least 6 months. ii) Orthotopically-placed pig heart grafts survive and function fairly consistently (eg, 7 of 10) for >3 months, with some primates surviving >6 months (Cooper et al., 2000). iii) Absence of life-threatening consumptive coagulopathy (Buhler et al., 2000; Lin et al., 2009; 2010). 4. Low incidence of immunosuppression-related complications, such as infection and malignancy (Teotia et al., 2005). They also insisted that patient selection must be considered. In the heart, the left ventricular pressure by telemetry proved to be the most valuable parameter to assess graft heart function in pig-to-primate models (Horvath et al., 2010).

Porcine renal xenotransplantation

Kirkeby and Mikkelsen (2008) reported the distribution of the alphaGal- and the non-alphaGal T-antigens in the pig kidney, suggesting the potential targets for rejection in pig-to-man xenotransplantation. Kidneys from normal pigs were almost rejected rapidly by antibody-mediated complement injury. In HAR, the recognition of pig antigens, predominantly Gal α 1,3Gal (Gal), by primate natural antibodies leads to complement activation, resulting in extensive intravascular coagulation and thrombosis, endothelial injury, interstitial hemorrhage and edema, and

infiltration of polymorphonuclear leukocytes into the tissues (Shimizu and Yamada, 2006). An attempt to eliminate hyperacute rejection with human and baboon antibody in GalT-KO kidney has been tried (Diswall et al., 2010). Chen et al. (2005) reported that acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. They also suggest that non-Gal antibodies are associated with the development of acute humoral xenograft rejection of hDAF transgenic porcine kidneys in baboons receiving anti-Gal antibody neutralization therapy (Chen et al., 2005). Renal endothelial heterogeneity accelerates AVR in pig-to-baboon xenotransplantation, compared to heart xenografts (Knosalla et al., 2009). With full immunosuppression, there was deposition of IgM, IgG, and complement, and neutrophil and macrophage infiltration, but there was minimal T and B cell infiltration, and no evidence of a T-cell-dependent elicited antibody response, extending the kidney xenograft to 3 days (Ezzelarab et al., 2009). Recently Lin et al. (2010) reported that prevention of recipient platelet activation may be crucial for successful pig-to-primate kidney transplantation. The challenges for the prevention of human and baboon blood immune responses in kidney transplantation have been attempted (Diswall et al., 2010).

Pancreatic islet xenotransplantation

High yields of pure and viable porcine islet cells to be used for microencapsulation are crucial for successful xenotransplantation and the reduction of the damage in porcine islet isolation should be considered (Stiegler et al., 2010). Islet transplantation into the portal vein is the current clinical practice. This method can induce the dysfunction in islet engraftment and survival owing to low oxygen tension, an active innate immune system, and the provocation of an inflammatory response, eventually resulting in the loss of many transplanted islets. Thus, subcutaneous transplantation has been recommended as an alternative choice (van der Windt et al., 2008).

Adult and neonatal pig islets xeno-transplanted in immunosuppressive nonhuman primates survived for more than 6 months (Cardona et al., 2006; Hering et al., 2006). These reports are encouraging since porcine pancreatic xenograft can survive longer than other xenograft organs, suggesting the crucial clue in xenotransplantation. Several investigators alleged that xenotransplantation of pig pancreatic primordia may be a candidate model as a therapeutics for both types 1 and 2 diabetes (Thomas et al., 1995; Rogers et al., 2006). Rogers et al. (2007) demonstrated that transplantation of embryonic day (E) 28 (E28) pig pancreatic primordia into the mesentery of STZ-diabetic rhesus macaques reduced insulin requirement, suggesting the availability for diabetes. Although the report

of porcine pancreatic islets xenograft into non-human primates was restricted to type I diabetes (van der Windt et al., 2009; Cooper and Casu, 2009), the usefulness in type II diabetes also remains to be attempted. Komoda et al. (2005) have demonstrated that islets from transgenic pigs expressing N-acetylglucosaminyltransferase-III showed prolonged survival after transplantation into cynomolgus monkeys.

PORCINE VIRUS INFECTION

Substitution of human organs to pig organs raises concerns about the risks of transfer of infectious agents from the pig cells to xenotransplantation recipients. Among many viruses, porcine endogenous retroviruses (PERVs) are important candidate to these problems. A major concern in pig-to-human xenotransplantations is the potential risk of transmission of PERVs integrated in the pig genome. Careful selection and/or genetic-engineering of pig herds should aid in minimizing the risk of PERVs infection and/or pathogenicity. Two types of PERV are present in pigs, human-tropic PERV-A and PERV-B, which are both present in the genome of all pigs, and PERV-C, which is not ubiquitous and infects only pig cells (Denner et al., 2009). Yu et al. (2009) indicated that a short-term and long-term PERV infection of HEK-293 cells *in vitro* does not result in any significant changes in host cells as well as in PERV LTR sequence.

The major strategies used to reduce hyperacute rejection such as depletion of anti-Gal antibodies and genetic engineering of swine to express human complement regulatory proteins to decrease complement deposition, might impact host defenses against viral infection (Meije et al., 2010). Increased levels of circulating PERV virus have not been detected in the GalT-KO swine or in immunosuppressed baboon recipients of GalT-KO grafts (Issa et al., 2007). To reduce the risk of PERV infection in xenograft recipients, diverse strategies are attempted, including use of nontransmitting swine or swine without active PERV loci as source animals, use of antiretroviral agents in recipients, viral vaccines, or the reduction of viral replication *in vitro* using RNA interference, various antibody therapies and amplification of antiviral restriction factors (Dieckhoff et al., 2007; Meije et al., 2010). In addition to PERV, porcine cytomegalovirus, herpesvirus, and hepatitis E virus also should be controlled since these viruses are common in immune-suppressed recipients of allotransplants (Mueller et al., 2004; Kamar et al., 2008).

CONCLUSION

In this review, we described the basic concept of the

rejection in pig-to primate xenotransplantation. The rejection of porcine xenotransplantation should be solved before clinical application to human. We also discussed a number of genetic engineered pig models used in xenotransplantation, and several important organs in porcine xenotransplantation. Further research for genetic modification of the organ-source pig will be prosperous. These may be associated with the identification of pig non-Gal antigens that are targets for natural or elicited antibodies, natural killer cells, and/or macrophages. Although genetically-modified pigs have overcome the problem of HAR, coagulation dysregulation between species remains an important challenge. In addition, the risks of the approaches to cell function and to the health of the recipient should be carefully evaluated. It is important to emphasize that the escape from PERV is essential for the complete success of porcine xenotransplantation into non-human primates before human clinical trials. It is generally recognized that success of xenotransplantation into non-human primate model should be preceded prior to human clinical application. Therefore, all efforts to solve many obstacles in porcine xenotransplantation should probably be performed.

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