



The potential of genetically-engineered pigs in providing an alternative source of organs and cells for transplantation

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Abstract

There is a critical shortage of organs, cells, and corneas from deceased human donors worldwide. There are also shortages of human blood for transfusion. A potential solution to all of these problems is the transplantation of organs, cells, and corneas from a readily available animal species, such as the pig, and the transfusion of red blood cells from pigs into humans. However, to achieve these ends, major immunologic and other barriers have to be overcome. Considerable progress has been made in this respect by the genetic modification of pigs to protect their tissues from the primate immune response and to correct several molecular incompatibilities that exist between pig and primate. These have included knockout of genes responsible for the expression of major antigenic targets for primate natural anti-pig antibodies, insertion of human complement- and coagulation-regulatory transgenes, and knockdown of swine leukocyte antigens that stimulate the primate's adaptive immune response. As a result of these manipulations, the administration of novel immunosuppressive agents, and other innovations, pig hearts have now functioned in baboons for 6-8 months, pig islets have maintained normoglycemia in diabetic monkeys for > 1 year, and pig corneas have maintained transparency for several months. Clinical trials of pig islet transplantation are already in progress. Future developments will involve further genetic manipulations of the organ-source pig, with most of the genes that are likely to be beneficial already identified.

Keywords: pig, blood transfusion; pig, genetic-engineered; pig, islets; pig, organs; xenotransplantation

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INTRODUCTION

There is a critical shortage of organs, cells, and corneas from deceased human donors worldwide. Furthermore, there are also shortages of human blood for transfusion. These shortages have been exacerbated in several countries by the increased incidence of HIV positivity in the population. A potential solution to all of these problems is the transplantation of organs, cells, and corneas from a readily available animal species, such as the pig, and the transfusion of red blood cells from pigs into humans. However, to achieve these ends, major immunologic and other barriers have to be overcome.

PIG HEART AND KIDNEY XENOTRANSPLANTATION

The primate immune response to a transplanted pig organ

Studies in the 1980s demonstrated that the primate

immune response to a transplanted pig organ was dramatically rapid, with hyperacute rejection occurring generally within minutes^[1]. If this was prevented by various approaches, a delayed form of humoral rejection occurred within a few days. Both of these responses were related to human IgM and IgG antibody binding to the pig cells, in particular to the vascular endothelium, activating the complement and coagulation cascades as well as innate immune cells, resulting in thrombosis, vascular endothelial disruption, and interstitial hemorrhage^[2].

There was also evidence from in vitro studies that the primate T cell response to a pig organ was at least as strong as the response to an allograft, but in vivo the adaptive immune response is rarely obvious because it is overwhelmed by the humoral response.

Approaches to overcome the primate immune response

The removal of antibodies from the primate's blood, e.g., by plasmapheresis or anti-pig antibody

Table 1 Genetically-modified pigs currently available for xenotransplantation research*

Pig model	Modified genes
Gal antigen deletion or 'masking'	α 1,3-galactosyltransferase gene-knockout (GTKO) Human H-transferase gene expression (expression of blood type O antigen) Endo-beta-galactosidase C (reduction of Gal antigen expression)
NonGal antigen deletion	N-glycolylneuraminic acid (NeuGc-KO) + α 1,3-galactosyltransferase gene-knockout (GTKO)
Complement regulation by human complement-regulatory gene expression	CD46 (membrane cofactor protein) CD55 (decay-accelerating factor) CD59 (protectin or membrane inhibitor of reactive lysis)
Anticoagulation and anti-inflammatory gene expression or deletion	Human tissue factor pathway inhibitor (TFPI) Human thrombomodulin Human CD39 (ectonucleoside triphosphate diphosphohydrolase-1) Von Willebrand factor (vWF)-deficient (natural mutant) Human endothelial protein C receptor (EPCR)
Suppression of cellular immune response by gene expression or downregulation	Porcine CTLA4-Ig (Cytotoxic T-Lymphocyte Antigen 4 or CD152) LEA29Y (Inhibition of the B7/CD28 co-stimulatory pathway of T-cell activation) CIITA-DN (MHC class II transactivator knockdown, resulting in swine leukocyte antigen class II knockdown) Human TRAIL (tumor necrosis factor-alpha-related apoptosis-inducing ligand) HLA-E/human β 2-microglobulin (inhibits human natural killer cell cytotoxicity) Human CD47 (for species-specific CD47-SIRPalpha natural interaction on macrophages) Human FAS ligand (CD95L) Human GnT- III (N-acetylglucosaminyltransferase III) gene
Anticoagulation, anti-inflammatory, and anti-apoptotic gene expression	Human A20 (tumor necrosis factor-alpha-induced protein 3) Human heme oxygenase-1 (HO-1) Human TNFRI-Fc (tumor necrosis factor-alpha receptor I-Fc)
Prevention of porcine endogenous retrovirus (PERV) activation	PERV siRNA

* Modified from Cooper DKC, et al.^[10]

Pigs with combinations of genetic modification, e.g., GTKO with added transgenes, are available.

absorption, resulted in a delay of the humoral response for hours or days, but, with the return of antibody and the infiltration of innate immune cells, delayed rejection inevitably occurred^[3]. Agents that depleted complement or blocked its activation, e.g., cobra venom factor, were effective in preventing hyperacute rejection, but were eventually overwhelmed by other immune mechanisms resulting from antibody binding, activation of the vascular endothelium, and innate immune cell activity. Pharmacologic immunosuppressive agents, which were highly effective in preventing the rejection of allografts, had almost no effect in delaying the rejection of a xenograft.

Genetic modification of the organ-source pig

The most significant steps toward overcoming the primate humoral response have been through the development of genetically-engineered pigs. This initially took two forms. First, human complement destruction of the pig vascular endothelium was inhibited by the introduction of human complement-regulatory genes into the pig, e.g., CD46, CD55 and CD59. The first of these was CD55 (decay-accelerating factor, DAF), which largely protected the pig cells from the action of complement^[4]. Second, the important galactose- α 1,3-galactose (Gal) oligosaccharide antigens on the surface of the pig vascular endothelial cells were deleted by gene-knockout technology, resulting in α 1,3-galactosyltransferase gene-knockout (GTKO) pigs that do not express these antigens^[5,6].

This two-pronged approach, i.e., deletion of the most important antigen (Gal) and blockade of human complement activation, enabled significant progress to be made. Transplantation of pig organs was extended from minutes, hours, or days to weeks or even months^[7]. However, it was observed that a thrombotic microangiopathy developed in the graft^[8], resulting in ischemic injury, frequently associated with the development of a consumptive coagulopathy that could be life-threatening to the recipient^[9]. Thrombotic microangiopathy was particularly obvious in pig heart grafts, but less obvious in kidney grafts, where consumptive coagulopathy developed more rapidly.

The development of thrombotic microangiopathy is almost certainly related to a low-grade immune response initiated by binding of antibodies directed to 'nonGal' antigens on the graft vascular endothelium, coupled with molecular incompatibilities between the coagulation-anticoagulation systems of primate and pig. There are several discrepancies in this system, e.g., relating to the interaction of primate thrombin and pig thrombomodulin^[9,10]. Attempts to overcome this dysregulation include the introduction into the organ-

source pig of human coagulation-regulatory (anticoagulant or anti-thrombotic) genes, e.g., thrombomodulin, tissue factor pathway inhibitor and CD39 (**Table 1**).

There is also evidence of an inflammatory response to the pig organ and this again may be overcome by the introduction of human anti-inflammatory genes, e.g., hemoxygenase-1 and/or A20. Furthermore, several of the anticoagulant genes have anti-inflammatory effects.

It should be noted that there is increasing evidence of interaction between the immune response and coagulation and inflammatory responses^[11]. For example, it has been demonstrated that thrombin activates a T cell response to the transplanted pig organ.

Advances in immunosuppressive therapy

A second reason for the improvement in outcome of pig xenografts is the introduction of novel immunosuppressive agents, such as T cell costimulatory blockade molecules. Although these have not played as important a role as genetically-engineered pigs, they have increased success in overcoming the adaptive immune response, thus preventing T cell infiltration in the graft and the production of T cell-dependent anti-pig antibodies. However, the continued presence of low levels of natural anti-nonGal antibodies in the primate remains problematic.

Here again, genetic engineering of the source pigs directed towards overcoming the adaptive immune response is proving beneficial. Pigs that express the costimulatory blockade agent, CTLA4-Ig, have been produced^[12], with highly effective results on in vitro testing^[13]. However, the production of soluble CTLA4-Ig by the pig tissues was so good that the pigs developed infectious complications, preventing their long-term survival. Attempts to utilize this technology have been successful by expressing the agent only in specific cells, e.g., pancreatic islets using an insulin promoter^[14].

As swine leukocyte antigen (SLA) class II plays such a major role in the T cell response to a transplanted organ, pigs have also been developed in which a mutant human class II transactivator has been introduced, thus reducing expression of SLA class II and, in particular, minimizing its upregulation when the primate immune response is activated^[15]. These approaches should enable the level of exogenous immunosuppressive therapy administered to the recipient of a pig graft to be significantly reduced.

Pig liver and lung xenotransplantation

Through a number of possible mechanisms, after pig liver xenotransplantation the recipient's platelets are removed from the circulation within minutes or hours, inducing a state of thrombocytopenia which

results in spontaneous bleeding^[9]. There are approaches through genetic engineering of the pig that could overcome this problem. Pig lungs appear to be particularly susceptible to immediate coagulation and complement cascade injury, which currently causes graft destruction within hours. Graft survival is steadily being prolonged by transplanting lungs from pigs with multiple genetic manipulations^[10].

Pig islet xenotransplantation

Pancreatic islet allotransplantation is producing encouraging results in patients with type 1 diabetes. Pig insulin was administered successfully to such patients for many years as it differs from human insulin by only one amino acid. Whereas the number of deceased human donors is very limited, pigs as sources of islets would be unlimited.

However, the infusion of pig islets into the portal vein of the recipient (the current approach) is followed by a major destruction of islets from what has been termed the “instant blood-mediated inflammatory reaction” (or IBMIR)^[16]. This is believed to be a form of immune response that involves coagulation and complement pathways, and therefore can be considered to some extent the equivalent of hyperacute rejection. It results in insufficient numbers of islets remaining viable to maintain a state of normoglycemia in diabetic nonhuman primates.

Once again, the genetic engineering of pigs is playing a role in overcoming this problem. Islets from pigs expressing a human complement-regulatory protein, CD46, with or without certain other human proteins, e.g., CD39, tissue factor pathway inhibitor, have maintained a state of normoglycemia in diabetic monkeys for periods in excess of one year^[16], indicating that pig islet transplantation will eventually become clinically successful. The same immunosuppressive regimens that have proved successful in pig organ transplantation in nonhuman primates appear equally successful when islets are transplanted

Pig neuronal cell transplants have also been associated with significant success in studies in nonhuman primates with a Parkinson-like disorder^[17].

PIG CORNEAL XENOTRANSPLANTATION

There is a worldwide shortage of corneas from deceased human donors for purposes of transplantation, particularly in Asia and Africa^[18]. The transplantation of pig corneas may resolve this problem. Possibly because the cornea is relatively immunoprivileged, even wild-type pig corneal transplants have been followed for several weeks or months without rejection, but it is much more likely that corneas

from genetically-engineered pigs will be utilized. In vitro studies indicate a markedly reduced immune response to some of the genetically-engineered pig corneas currently available^[19]. Decellularization of the pig cornea (with subsequent repopulation by recipient cells) also reduces immunogenicity. Pig corneal xenotransplantation should reach the clinic in the relatively near future.

PIG RED BLOOD CELL XENOTRANSFUSIONS

Even in western countries with sophisticated blood transfusion services, there are periods when there are shortages of blood. GTKO pigs, all of blood type O (the universal donor), can be produced in unlimited numbers. In vitro data indicate that their red blood cells are protected from the immune response more successfully than ABO-incompatible human red blood cells, but not as yet to the extent of the minimal response to human ABO-compatible red cells^[20]. This is almost certainly due to the presence of nonGal antigens that remain on the red cells after deletion of Gal. With the current techniques of genetic engineering, human transgenes, e.g., complement-regulatory proteins, are not expressed in red blood cells, and therefore new technologies will need to be developed. When this problem is resolved, it is quite possible that pigs known to be free of designated infectious microorganisms, will provide a much preferred source of red blood cells for transfusion than the average human donor.

CONCLUSION

Future developments will involve further genetic manipulations of the organ-source pig. Most of the genes that are likely to be beneficial are already known, but it is a matter of obtaining sufficient financial support to enable multiple genes to be introduced into the pigs, which is a time-consuming process. Antigenic targets for anti-nonGal pig antibodies are not yet certain, although it is clear that humans (but not nonhuman primates) have antibodies directed to the sialic acid, *N*-glycolylneuraminic acid (NeuGc), a carbohydrate antigen that is not expressed on human cells^[21]. This antigen may therefore require deletion before clinical trials can be fully successfully undertaken, though this remains uncertain. Pigs expressing neither Gal nor NeuGc have recently been produced^[22].

If ubiquitous high expression of a gene is determined to be detrimental to the pig's health, it is possible that gene expression will need to be transient. If expression is present only in the organ after transplantation, it would not be detrimental to the recipient.

Techniques are available to switch on or switch off gene expression, but these are not yet currently employed in organ-source pigs.

The ultimate goal will be to develop a state of immunological tolerance whereby the recipient would no longer attempt to reject the transplanted organ. This may be particularly important because, even if hyperacute, delayed humoral, and acute cellular rejection can be fully overcome, graft atherosclerosis in the form of chronic rejection may develop through the continued presence of a low level of anti-nonGal antibody. It is possible that only the induction of a state of tolerance will allow truly long-term survival of pig organ grafts. The potential to achieve this is high once the current problems have been overcome.

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