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The potential hazards of xenotransplantation: an overview

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Summary
Xenotransplantation, in particular the transplantation of pig cells, tissues and organs into human recipients, may alleviate the current shortage of suitable allografts available for human transplantation. This overview addresses the physiological, immunological and microbial factors involved in xenotransplantation. The issues reviewed include the merits of using pigs as xenograft source species, the compatibility of pig and human organ physiology, and the rejection mechanism and attempts to overcome this immunological challenge. The authors discuss advances in the prevention of pig organ rejection through the creation of genetically modified pigs, more suited to the human micro-environment. Finally, in regard to microbial hazards, the authors review possible viral infections originating from pigs.

Keywords
Alpha1,3-galactosyltransferase gene knock-out – Endogenous retrovirus – Genetically modified pig – Pig tissue – Pig – Rejection – Xenotransplantation.

Introduction

Xenotransplantation is the transfer and implantation of cells, tissues and organs from one species into another. Xenotransplantation in humans is defined by the United States Food and Drug Administration as: 'any procedure that involves the transplantation, implantation, or infusion into a human recipient of either live cells, tissues, or organs from a nonhuman animal source, or human body fluids, cells, tissues or organs that have had ex vivo contact with live nonhuman animal cells, tissues or organs' (105). Thus, clinical xenotransplantation can include:

a) the transplantation of solid animal organs, e.g. the heart, kidney, liver

b) the transplantation of live animal cells, e.g. neuronal and pancreatic islet cells

c) the use of viable animal cells and/or organs as part of a medical device, e.g. extra-corporeal liver or kidney perfusion.

In the 1960s, the high mortality rates due to organ failure led to attempts at both xenotransplantation and allotransplantation. Details of these xenotransplantation procedures have been listed in a previous review (55), but no cases of survival after one year were reported for either the xenograft or the patient. In contrast to the disappointments encountered during xenotransplantation, many disease treatments have achieved success with allotransplantation, and long-term prognoses are often encouraging. Demands for human donor organs and tissues have therefore increased dramatically and cannot be met by materials from cadavers or living donors (United Network for Organ Sharing website: http://www.unos.org/data/). For this reason, xenotransplantation has attracted renewed interest as an alternative to allotransplantation.

Xenotransplantation has the following advantages in comparison with allotransplantation:

– it provides an unlimited and predictable organ supply

– it allows for advanced planning and elective surgery

– it allows for immunological pre-treatment, if required

– organs are harvested at the time they are required

– breeding specific-pathogen-free (SPF) source animals minimises the risk of exposure to pathogens

– organs can be screened for infection before harvesting.

Although non-human primates (NHP) would seem to be the most obvious choice as source animals for xenotransplantation, pigs are currently the favoured species for this role, despite their many dissimilarities to humans. Table I summarises the problems with using NHP and the advantages of using pigs. Three main obstacles must be overcome for pig-to-human xenotransplantation to be successful:

– physiological incompatibility

– immunological rejection

– microbial infections (zoonoses).

Physiological incompatibility

The wide phylogenetic disparity between pigs and humans may preclude xenotransplantation, due to inherent physiological incompatibility. There is, as yet, insufficient information about the way in which animal organs will function once they have been transplanted into human recipients. For an animal xenograft with a predominantly mechanical function, e.g. the heart pumping blood or the lungs oxygenating blood, physiological compatibility in a human recipient may be easier to achieve. However, if the xenograft performs complex biochemical and metabolic functions, such as the kidney or liver, species differences may lead to physiological incompatibility (38, 91).

For example, renal xenografts require the proper functioning of erythropoietin. Porcine erythropoietin is known to be non-functional in humans as the pig hormone is not recognised by human erythropoietin receptors on red blood cell precursors within the bone marrow (91). It would be necessary either to genetically modify the porcine xenograft to overcome this deficiency, or to supplement the xenotransplantation procedure with an independent corrective procedure.

In contrast, certain porcine products have been found to be physiologically compatible with the human system, such as the porcine plasma-derived factor VIII and insulin. Porcine insulin is an adequate substitute for human insulin in type 1, insulin-dependent diabetes. Consequently, the transplantation of pig-insulin-producing beta cells (β-cells) may be used to treat type 1 diabetics. Porcine islet-like cell clusters (ICC) from foetal pig pancreases which were transplanted into diabetic nude mice were able to differentiate into insulin-releasing β-cells. When porcine ICC were transplanted into type 1 diabetics, the xenografts survived for several months and released insulin, as demonstrated by the detection of the C peptide in the urine of the recipients. The C peptide is a substance which is also released by β-cells in a molar ratio of 1:1 to insulin. However, in this case, the porcine insulin appeared to have no effect in the recipients (36). In a more recent human trial in Mexico City, twelve type 1 diabetic adolescents were implanted with insulin-producing pancreatic pig cells. It is reported that one child has stopped having insulin injections, while five others have reduced insulin requirements (16).

Table I
The reasons why pigs are favoured over non-human primates as a source species for xenotransplantation

Advantages of using pigs	Disadvantages of using non-human primates
Pigs attain sexual maturity within 9 months	Non-human primates are slow to attain breeding maturity
Pigs have short gestation periods (3.5 months)	Non-human primates have a long gestation period
Pigs have large litters of between 6 and 16 piglets	Non-human primates usually have only a single offspring
Large-scale pig-breeding and farming are common	Large-scale farming of non-human primates is difficult
There are no specific ethical issues with using pigs	Non-human primates have intellectual and social natures very similar to those of humans, making their use unethical
Pigs are not an endangered species	Chimpanzees are currently considered an endangered species
The organ size and life expectancy of an adult pig (approximately 30 years) are compatible with those of adult humans	Certain human pathogens grow in primate cells but not in pig cells (e.g. hepatitis viruses)

Immunological rejection

To date, attempts to control the immune response of the host and prevent xenograft rejection have not been successful. The various types of rejection have been categorised according to their reaction times (4):

a) hyperacute rejection (HAR) occurs within minutes of transplantation

b) acute vascular rejection (AVR)/delayed xenograft rejection may begin 24 hours post transplantation (when HAR has been prevented)

c) cell-mediated rejection may occur in vascularised grafts when both HAR and AVR have been averted.

The recent production of genetically engineered animals in an attempt to prevent HAR has been heralded as a major technological advance. In this paper, the authors focus on HAR and animal engineering. An extensive, detailed review on immunological rejection has already been undertaken (4).

In NHP models of clinical xenotransplantation, HAR destroys the vascular endothelium of the xenograft, analogous to the case of a mismatched human allograft across the ABO blood group barrier. Hyperacute rejection is primarily mediated through complement activation, once the pre-formed antibodies of the host bind to the xenograft (pig) antigens. The major xenograft epitope is a disaccharide residue, galactose-α (1-3)-galactose (α-Gal), expressed in all mammals except humans, apes and Old World monkeys. This species difference is based on the glycosylation activity of the enzyme α1,3-galactosyl transferase (α(1-3)GT). Humans possess only a non-functional pseudogene for this enzyme and consequently develop anti-αGal antibodies, raised in response to antigenic stimulation to α-Gal epitopes expressed by gastro-intestinal tract bacteria (34). Up to 5% of human plasma immunoglobulin M (IgM) is directed to the α-Gal antigen (54), which triggers the classical complement cascade leading to endothelial cell lysis. The onset of HAR occurs before the death of the endothelium and involves endothelial cell activation. This is referred to as 'type I' endothelial activation and is responsible for HAR manifestations such as intravascular thrombosis and extravascular haemorrhage and oedema (4).

Both short and long-term strategies have been implemented to overcome HAR. Short-term measures include altering the xenograft recipient by depleting naturally occurring anti-αGal antibodies (52, 113). Extracorporeal perfusion of NHP blood through an α-Gal adsorption column, or intravenous infusion of the recipient with an α-Gal oligosaccharide, and the administration of cobra venom factor or soluble complement receptor type 1 have all been attempted (49, 79, 81, 115). While these therapies certainly prolong xenograft survival, they have not been sufficient to prevent the eventual loss of the xenograft. Thus research turned its focus towards engineering the animal source.

Transgenic pigs have been produced which express high levels of the enzyme H-transferase (α1,2-fucosyltransferase), which competes with α(1-3)GT for a common acceptor substrate (21, 83, 90). Human complement regulatory proteins (CRP) are a family of genetically and structurally related proteins, including cluster of differentiation antigen (CD) 46 (membrane co-factor protein), CD55 (decay-accelerating factor) and CD59. It is essential that all genetic modifications produce animals that are healthy and thrive. Complement regulatory proteins are thought to function in a species-specific manner (25, 46, 64, 69). Thus, the over-expression of CRP can both leave the immune status of the pig unaffected and down-regulate the activation of complement upon xenotransplantation, thus preventing graft HAR (3). Transgenic pigs expressing human CRP have since been generated and organs from such pigs have been tested in NHP (8, 14, 15, 21, 22, 23, 26, 53, 85, 86, 116, 117).

However, the use of pigs that are transgenic for human CRP may cause unintended consequences. First, certain viruses are known to incorporate cell surface molecules into their envelopes while budding from the cell (61, 67, 82, 94, 95). Therefore, viruses such as retroviruses budding from transgenic pig cells are likely to incorporate human CRP in their envelopes. This may render the viruses resistant to human complement-mediated inactivation and consequently increase the susceptibility of human xenograft recipients to viral infection. Porcine endogenous retroviruses (PERV), produced through a pig cell line engineered to express human CD59, were found to incorporate the human protein into their envelopes, which inhibited complement-mediated lysis of the virus. However, in this study, human serum still neutralised PERV infectivity efficiently (93). More recently, the authors have shown that enveloped rhabdoviruses can incorporate human CD55, and that retroviruses and rhabdoviruses can be partially protected by this molecule when they are produced by pig endothelial cells transgenic for human CD55 (56).

Secondly, some human CRP can serve as receptors for human viruses. The Edmonston measles virus strain and vaccine strains derived from it use human CD46 for cellular entry, whereas wild-type measles viruses use signalling lymphocyte activation molecule (97). Echo, Coxsackie B (B1, B3 and B5) and enterovirus type 70 viruses enter cells through human CD55 (6, 7, 20, 29, 78, 88, 89, 107). Thus, pigs transgenic for CD46 or CD55 may become susceptible to those human pathogens.

Conversely, animal measles-related morbilliviruses and animal picornaviruses (e.g. swine vesicular disease) could pre-adapt to use human CRP for entry, which may allow their transmission into formerly resistant humans (108).

A more straightforward strategy, to 'knock out' the expression of α-Gal, and thus avoid HAR, has also been sought. The recent development of new pig cloning techniques by somatic cell nuclear transfer provides a means of disrupting or deleting genes (77). This technology, following on from the generation of αGal knock-out (KO) mice (99, 100), has been used to generate pigs without αGal. Pigs were initially 'knocked out' for one allele of the α(1-3)GT enzyme gene by gene-targeting (24, 47). More recently, double KOs, which do not express the enzyme, have been engineered (44, 75).

Alpha-Gal null cells were selected for resistance to a bacterial toxin binding to αGal (75) or baboon anti-αGal natural antibodies with complement (44) from fibroblast cultures of heterozygous aαGal +/– foetuses. Alpha-Gal null animals were generated by nuclear transfer using these fibroblast cells. These methods achieved the creation of αGal null animals from heterozygotes more quickly than natural breeding and also afforded different forms of αGal gene defects. Despite the complete removal of αGal, the animals appear healthy, at least in the first generation. However, future observation of these animals to detect any problems with fertility and long-term health will be very important, since such issues may affect both the donor animals and the long-term function of their organs, once transplanted. Organs from these animals have recently been used in NHP models of xenotransplantation. Dramatic improvements in survival have been observed, in comparison to results when using wild-type organs. Nonetheless, the organs are frequently lost, due to other immunological mechanisms, indicating that further optimisation of the immunotherapy is required (114). Alpha-Gal null animals, such as humans and NHP, produce natural anti-αGal antibodies and will therefore be useful in studying the effect of αGal in allotransplantation settings (28). As expected, enveloped viruses produced by αGal null cells are less sensitive to virus killing by human serum or anti-αGal natural antibodies with complement (56, 80), suggesting that a reassessment of the benefit-risk balance is required.

Active induction of tolerance, reviewed by Auchincloss and Sachs as well as Galili (4, 35), is an ambitious, long-term strategy combating both HAR and subsequent immunological responses. While mixed chimerism by transplantation of the bone marrow cells can reduce any immune response against xenografts autologous to the transplanted bone marrow cells, specific xenogeneic genes can be expressed in recipient cells by gene therapy technology. The α(1-3)GT gene has been introduced in αGal KO mice, which resulted in the induction of long-term tolerance and inability to produce anti-αGal antibodies (10, 11). Preliminary trials with the xenotransplantation of Gal KO porcine bone marrow have achieved hyporesponsiveness, but further work is required (103).

Zoonoses

The risk of zoonosis, i.e., the transfer of pathogens between species, is increased in xenotransplantation because normal host defences, including the skin and mucosal surfaces, are bypassed when human and animal tissues are placed in close contact. Animal pathogens that, under normal circumstances, would be non-infectious to humans may become infectious in a xenotransplantation scenario. The consequences of subsequent human-to-human transmission of these animal pathogens from transplant recipients to the general population could be profound, and the development of potential new epidemics is a major concern. The use of immunosuppressive therapy to minimise graft rejection further exacerbates the risk of infection from otherwise non-infectious or latent animal pathogens. The occurrence of zoonotic infections originating from pigs is not unknown. Fatal cases of 'swine influenza', which probably include the 1918-1919 influenza pandemic, have been attributed to the swine influenza virus (98, 109). Epidemics of encephalitis that occurred in Malaysia a nd Singapore were caused by Nipah virus in pigs. The virus had recently migrated from its reservoir in fruit bats (17, 18, 74).

Although most known pathogens can be eliminated by SPF breeding, in addition to careful screening and monitoring of source animals (92), the main risk of zoonosis arises from the transfer of source pathogens that are as yet unidentified, difficult to eliminate or maintained in a latent or intracellular state in an asymptomatic host. Furthermore, genetic modification of source animals or host tolerance induction may alter the susceptibility of the host to animal pathogens or 'humanise' animal pathogens, allowing them to survive in human recipients (108).

Viruses are currently under scrutiny as zoonotic agents. Viruses may be non-pathogenic in their animal host but could cause serious disease in humans and can be efficiently transmitted with viable cellular grafts. Retroviruses can be transferred to humans and have long latency periods during which they are able to spread in the population before an epidemic becomes apparent. For example, human immunodeficiency virus-1, the causative agent of the acquired immune deficiency syndrome pandemic, was discovered in the early 1980s. However, initial infections had occurred before 1960 and were followed by more than two decades of silent

human-to-human transmission (106, 118). The following types of viruses must be taken into consideration when assessing the safety of pigs as source animals:

– porcine endogenous retroviruses, which can infect human cells (70)

– other recently identified pig viruses (Table II)

– as yet unknown infectious agents.

For example, the swine hepatitis E virus (HEV), which is antigenically and genetically related to human HEV strains, can cross the species barrier under experimental conditions to infect NHPs. Furthermore, a human strain of HEV was found to infect SPF pigs (62, 63).

Porcine endogenous retroviruses

Endogenous retroviruses (ERV) are remnants of ancestral retroviral infections that have integrated into the germline deoxyribonucleic acid (DNA) as a proviral genome, which is vertically transmitted from parent to offspring. The provirus usually survives as part of the host genome rather than as an infectious agent. Over evolutionary time periods, most of these proviruses acquire mutations so that, with few exceptions, they become defective and incapable of producing protein. However, replication-competent ERV have been identified in several animal species, including chickens, mice, cats, primates and pigs (9, 71). Although ERV are normally non-pathogenic to their natural host, they have been shown to propagate efficiently and can cause disease if they cross species barriers. Gibbon ape leukaemia virus, for example, can cause leukaemia in captive gibbons. This virus, thought to have originated from an endogenous mouse virus (51), belongs to the same genus, gammaretrovirus, as PERV (57). Gibbon ape leukaemia virus also clusters phylogenetically with koala retrovirus, suggesting that these viruses are closely related and that recent cross-species transmissions have occurred (39).

Porcine endogenous retroviruses were first described in the 1970s as a C-type retrovirus containing a ribonucleic acid genome which hybridised to pig genomic DNA (2, 12, 50, 101). Later, in 1997, PERV infection of human cells was reported (70) and, since then, considerable effort has been put into the study of PERV. In this paper, the authors present a brief overview of PERV research, focusing on the progress made in the last two years. More detailed information can be found in Magre *et al.* (55).

Infectious PERV belong to the genus of gammaretrovirus and are classified as gamma (γ) 1 group, while other groups of beta- and gamma- retrovirus sequences have been found in pig genomes (31, 73). The γ 1 PERV group consists of three subgroups, A, B and C. These subgroups share homologous gag genes (which encode core structure proteins) and pol genes (which encode enzymes), but each subgroup has a distinctive env gene (which encodes envelope proteins directing virus-cell surface interaction and fusion), and therefore uses different receptors (1, 48, 96). Porcine endogenous retroviruses A and B can infect certain cells of human and pig origin, as well as cells originating in some other species, while the host range of PERV-C is restricted to pigs (96). The observations that pig primary cell cultures can produce PERV which infect human cells (58, 66, 111) and that human primary cell cultures are susceptible to PERV infection (59, 60) substantiated the potential risk of PERV zoonotic transmission in pig-to-human xenotransplantation. However, retrospective analyses on xenograft recipients have found no evidence for PERV infection (27, 41, 42, 68, 72, 76). These findings must be interpreted with caution because no long-term xenotransplantation of physiologically functional pig tissues or vascularised pig organs has been achieved or successful to date. Furthermore, treatment in the future to reduce immune response, such as the use of genetically engineered animals, will increase the risk.

Breeding or engineering animals with reduced PERV activity is thus desirable. Pigs have about 50 copies per genome of γ 1 PERV group provirus (1, 48, 70). While some pigs are devoid of PERV-C, all domestic pigs possess both PERV-A and B. Porcine endogenous retroviruses

Table II
Recently identified porcine viruses

Pig pathogen	Viral family	Pathogenesis in humans	References
Nipah virus	*Paramyxoviridae*	Viral encephalitis	18
Porcine cytomegalovirus	*Herpesviridae*	Transmission risk and pathogenesis unknown	19, 33
Porcine encephalomyocarditis virus	*Picornaviridae*	Fever, neck stiffness, lethargy, delirium, headaches, vomiting	13
Porcine hepatitis E virus	Unclassified; hepatitis E-like virus	Liver disease, hepatitis	37, 62, 110
Porcine lymphotropic herpesvirus types 1 and 2	*Herpesviridae*	Transmission risk and pathogenesis unknown	30, 104
Porcine rotavirus	*Rotaviridae*	Diarrhoea	84
Porcine torovirus	*Coronaviridae*	Transmission risk and pathogenesis unknown	45
Swine influenza virus	*Orthomyxoviridae*	Influenza	98, 109

A and B appear to have been introduced into pigs and pig-related species in Old World pigs, after they separated from New World peccaries (73, 102). It is unclear how many copies/loci of PERV provirus are infectious in each pig, or in the pig species in general, but most copies/loci seem to be defective. Extensive screening of pig genomic libraries has identified only a few loci that harbour PERV proviruses with intact open reading frames (43, 65). Some of these provirus clones were infectious, but their infectivity appeared to be much lower than that found in cell-line-derived PERV or recombinant PERV isolated from miniature swine animals (5, 65).

Primary peripheral blood mononuclear cell cultures from miniature swine, including the herds bred for xenotransplantation purposes, often produce high-titre, human tropic PERV (66, 111). These PERV have recombinant genomes, mostly derived from PERV-C but containing the PERV-A receptor binding domain, and they therefore use PERV-A receptors to infect cells. One of these high-titre recombinants was used to clone a human complementary DNA encoding PERV-A receptor. Moreover, PERV-A receptor genes have been identified in humans, baboons and pigs (32). The same recombinant was further characterised, showing that it has multiple genetic determinants for high titre in both PERV-A and PERV-C- derived sequences (5, 40). Since miniature swine genomes do not have such recombinant forms, and recombination points vary among isolates from different miniature swine individuals, such recombinants are likely to have arisen in individual pigs or their primary cultures (5, 66, 87). Recombination may be a simpler method for PERV to become more active than multiple point mutations. While some miniature swine animals produce human tropic PERV, some animals of certain inbred miniature swine families have failed to produce human tropic PERV in parallel experiments (66, 112). Further investigation into pig genomics will aid in understanding these differences in the production of human tropic PERV and help to identify/generate animals with no/reduced potential to produce human tropic PERV.

Conclusion

Xenotransplantation offers much promise and many patients hope for its success. Research and development towards clinical success, however, require the convergence of multiple scientific disciplines and substantial financial resources. The authors have witnessed significant advances in this field in recent years, such as developments in the biology of organ rejection, the generation of αGal KO pigs and the compiling of knowledge on PERV. Such progress is in itself significant and interesting as basic science.

What of the future? Clearly, a single breakthrough will not overcome all problems at once. It is now important to combine these multiple disciplines and to assess objectively the practicality of further developments in the field. Such assessments must include alternative therapies. For example, life-long immunosuppression following xenotransplantation may not be justifiable if an alternative therapy is available. In this respect, induction of host tolerance to xenografts might be essential. Successful clinical xenotransplantation is still some way off and requires further effort, but the authors believe that this is a challenge worth undertaking.

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Les risques potentiels de la xénotransplantation

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Résumé
Les xénotransplantations, et notamment les transplantations de cellules, tissus et organes de porc chez l'homme pourraient parer au manque actuel d'allogreffes adaptées disponibles pour les transplantations humaines. Cet article traite des facteurs physiologiques, immunologiques et microbiens impliqués dans les xénotransplantations. Les auteurs abordent l'intérêt de l'utilisation du porc comme espèce source de xénogreffes, la compatibilité physiologique des organes porcins et humains, les phénomènes de rejet et les tentatives visant à résoudre ce défi immunologique. Ils discutent des avancées en matière de prévention du rejet des organes de porcs par la création de porcs génétiquement modifiés, mieux adaptés au microenvironnement humain. Concernant les risques microbiens enfin, les auteurs passent en revue les infections virales possibles d'origine porcine.

Mots-clés
Inactivation du gène alpha-gal – Porc – Porc génétiquement modifié – Rejet – Rétrovirus endogène – Tissu porcin – Xénotransplantation.

Repaso general de los posibles peligros de los xenotrasplantes

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Resumen
Los xenotrasplantes, en particular el trasplante de células, tejidos y órganos porcinos a un receptor humano, pueden paliar la actual escasez de aloinjertos para el ser humano. Los autores hacen un resumen de los factores fisiológicos, inmunológicos y microbianos que pueden tener importancia en un xenotrasplante. También describen las ventajas de utilizar el cerdo como especie donante de xenoinjertos, la compatibilidad fisiológica de los órganos porcinos y humanos y el mecanismo de rechazo, así como los intentos de resolver este problema inmunológico. Examinan asimismo el adelanto que supone la creación de cerdos genéticamente modificados, mejor adaptados al ambiente celular del ser humano, para prevenir el rechazo de órganos porcinos. Por último, respecto a los riesgos microbiológicos, pasan revista a una serie de infecciones víricas que el cerdo puede transmitir.

Palabras clave
Cerdo – Cerdo genéticamente modificado – Gen 'knock out' a-gal – Rechazo – Retrovirus endógeno – Tejido porcino – Xenotrasplante.

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