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REVIEW

Xenotransplantation: an update on recent progress and future perspectives

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Summary

Currently, the number of patients awaiting transplantation is continuously increasing, and shortage of available deceased organ donors is the major limitation for organ and cell allotransplantation. Research to develop alternative sources of tissues is ongoing and xenogeneic organs or cells represent an attractive solution. This review focuses on recent progress achieved in this field, including the development of newly genetically modified animal donors and new immunosuppressive approaches. As xenotransplantation is moving closer to clinical application, future perspectives must establish guidelines to ensure that future clinical trials are carried out ethically and safely.

Introduction

Well before the dawn of clinical allotransplantation (alloTx), the first reports of animal tissue transplantation (Tx) into human recipient were published [1,2]. Since these first attempts of xenotransplantation (xenoTx) in 1894 [2], organ and cells alloTx have become the only recognized clinical approach of organ replacement therapy. Currently, the major limitation to the further development of alloTx is the availability of human organs. While new sources of organ or cells are actively developed (e.g. non-heart beating donors, living donors and marginal donors), others are still under investigation (e.g. xenoTx, stem cell technology and human cell lines). Among them, xenoTx appears a promising approach. Cloning of genetically modified pigs represents an important progress and xenograft survival up to 6 months has been reported in pig-to-nonhuman primate models. These results are bringing xenoTx closer to clinical application and it is urgent that international collaborations are established to ensure that future clinical trials are carried out ethically and safely. In this paper, we review these recent developments and discuss future perspectives of xenoTx.

Immunological barriers to xenotransplantation

Largely for logistic reasons, the pig has been identified as the most suitable donor animal. When transplanted into untreated humans or nonhuman primates, pig organs are rejected hyperacutely within minutes by antibody-mediated complement activation. Hyperacute rejection (HAR) is the result of this incompatibility between donor and recipient encountered in vascularized organ xenoTx [3,4]. HAR is characterized by the destruction of the xenograft parenchyma and vasculature immediately after reperfusion, resulting in widespread interstitial hemorrhage and thrombosis [3]. HAR is induced by naturally occurring antibodies reactive against donor antigens [3,5,6]. The major target antigen of human natural xenogeneic antibodies is the galactoseα1,3galactose (Gal) sugar residue present on cell surface of lower mammals and New World monkeys [3]. The presence of natural antibodies against Gal in human and Old World monkeys and

humans is in relation with evolutionary differences between species in the basic immune defense against bacterial pathogens [7]. The main components implicated in HAR are: xenoreactive antibodies, complement and endothelial cells. It has been shown that removal of xenoreactive antibodies could prevent HAR [3,5,7,8]. Complement also plays a crucial role in HAR, mainly through activation of the classical pathway by xenoreactive antibodies and directly through the alternative pathway without antibody binding. Complement depletion can be achieved by administration of various agents, e.g. cobra venom factor or soluble complement receptor-1, and allows prevention of HAR [9].

However, the persistence or return of anti-Gal antibody, and/or the development of newly-formed (elicited nonGal) anti-pig antibodies, eventually leads to what has been variously termed acute vascular rejection, delayed xenograft rejection, or acute humoral xenograft rejection. Delayed xenograft rejection represents a from of delayed HAR, when T cell-dependent sensitization has occurred and elicited xenograft-specific antibodies are produced [5,10].

Acute cellular xenograft rejection, which appears to differ to that seen in allograft rejection, is a relatively rare phenomenon as a sole entity in xenotransplant models and is frequently associated with acute humoral xenograft rejection [11]. Little is yet known of the precise nature of the acute cellular rejection that is anticipated to follow or of any subsequent chronic rejection, e.g. graft vasculopathy that may develop at long-term [12–17].

The ideal donor pig

In terms of breeding, rearing, cost and ethical consideration the pig is viewed as the species of choice [18]. While being an immunological discordant species to human [18], anatomical, physiological and biochemical characteristics between pigs and humans show some compatibility. Some anatomical and physiological differences could be of concern, such as heart morphology, lung working position and kidney metabolic pathways [19]. Even when biological similarities are present, xenoproteins may be less effective than alloproteins [20]. Finally, pigs have another advantage over non-human primates as potential donors for human xenoTx, as they can be quite easily genetically manipulated unlike primates to express extrinsic genes, which could address some of the barriers to xenoTx [21].

Pigs transgenic for the human decay accelerating factor (hDAF) gene have been developed in the early 1990s, and the initial reports using them as donor for xenoTx in nonhuman primates showed improved results in terms of graft survival. hDAF is a human complement regulatory gene, which prevents activation of complement when these organs are exposed to human complement and offers protection against HAR [22]. However, hDAF organs did not survive in nonhuman primates over 3 months [23] and Cozzi *et al.* [24] recently demonstrated that hDAF pig organs transplanted in primates receiving intense immunosuppression were still rejected by humoral mechanisms [3,24,25]. More recently, it has also been shown that use of CD46 transgenic pig organs were able to protect grafts from HAR in baboons receiving no immunosuppression [26].

A new era – the α1,3-galactosyltransferase gene-knockout (GT-KO) pig

The birth of the first homozygous GT-KO pigs, not expressing the major xenoantigen recognized by human natural anti-Gal antibodies, was reported by PPL Therapeutics and the Pittsburgh team in 2003 [27]. Immerge Biotherapeutics also announced the production of GT-KO pigs and, in collaboration with the Massachusetts General Hospital group, presented the first *in vivo* results following the transplantation of these pig organs into baboons at the American Transplant Congress in Boston, in May 2004 [28]. GT-KO pig hearts were transplanted heterotopically in immunosuppressed baboons. The immunosuppression consisted of anti-human thymocyte globulin as induction, followed by maintenance therapy combining a human anti-CD154 monoclonal antibody, mycophenolate mofetil, and methylprednisolone. To prevent thrombotic complications, heparin was administered continuously in combination with aspirin. The mean organ xenograft survival was around 80 days, but some hearts survived up to 180 days, demonstrating clearly that these newly modified pig organs offer a significant progress in term of graft survival. Thrombotic microangiopathy occurred in several xenografted hearts, indicating that remaining coagulation disturbances have to be solved in order to allow long-term survival. Further genetic modifications allowing control of coagulopathy should further improve results and new clinical trials could be initiated again in a close future.

The production of heterozygous GT-KO pigs transgenic for human α1,2-fucosylosyltransferase has also been reported, with the aim of producing homozygous Gal-deficient animals expressing the H antigen [29]. These knockout and transgenic pigs might remove the need both for the expression of hDAF and the administration of soluble Gal glycoconjugates.

Physiological incompatibilities

A number of molecular incompatibilities have been identified between pig and human. The physiological and

biochemical variations that exist between these species include blood viscosity, liver metabolism, enzymes and hormones. Of particular concern has been the incompatibility of coagulation factors that might lead to the development of a pro-coagulant state in the graft with subsequent thrombosis. Genetic engineering approaches might be considered to overcome these barriers.

Coagulation disturbances are encountered in discordant model of organ and cells xenoTx [4]. These abnormalities are related to disparity of hemostasis pathways between discordant species [3,19,30–32]. While some of these coagulation disorders related to coagulation and platelet physiology incompatibilities have been shown to be inhibited by heparin derivatives, antithrombin III and prostacyclin inhibitor [3,31], more research is required to identify key factor of these processes [4,19,31]. Coagulation pathways between pig and human seems to bare incompatibilities which have to be resolved prior to initiation of new clinical xenoTx [3,19,30–32].

It is known that exposure of allogeneic islets to human blood triggers an inflammatory reaction leading to activation of coagulation [33], and this phenomenon seems to be amplified in islet xenoTx [32,33]. This phenomenon has been investigated by Hawthorne *et al.* [32] and Goto *et al.* [34] who have confirmed that pig islet cell clusters trigger an immediate blood inflammatory reaction in human blood, which could be prevented by either heparin, recombinant human antithrombine or dextran sulfate [32,34].

Experimental cellular xenotransplantation

Several cells xenoTx models are under investigation, among them islet xenoTx being the most active [33]. Shortage of human pancreases available for whole organ or islet alloTx, has lead to search for xenogeneic sources of islets. As porcine insulin has been used successfully in diabetic patients for decades, and is very similar to human insulin, porcine islets have been chosen as the most suitable source [35,36]. However, porcine islet isolation still remains challenging and is one of the key issues for future clinical application [37]. Brandhorst *et al.* [38] have shown that use of newly available collagenase preparations which enable adjustment of the neutral protease activity, porcine islet isolation improved porcine islet isolation in term of islet yields.

Some recent experiments have demonstrated the importance of co-stimulatory blockade to prevent islet xenograft rejection or even promote long-term graft acceptance. Long-term survival of discordant xenogeneic islets in mice model could be achieved with anti-CD154 monoclonal antibodies or CTLA4-Ig fusion proteins [39]. Bucher *et al.* [40] have demonstrated that discordant islet xenoTx in mice treated with anti-CD154 monoclonal antibody (MR1) resulted in long-term islet survival and prevented recipient sensitization to donor antigens.

Newly available immunosuppressive drugs, such as FTY720 and everolimus have been evaluated for their potential use in islet xenoTx. Hering *et al.* [41] have recently presented results at the American Transplant Congress in Boston in May 2004, reporting prolonged porcine islet graft survival in nonhuman primates treated by basiliximab, everolimus, FTY720 and anti-CD154 monoclonal antibody with insulin-independence >100 days. These data indicate that co-stimulatory blockade combined to clinically used drugs allow long-term survival of xenogeneic cells in a pre-clinical nonhuman primate model without significant morbidity.

Immunoisolation by encapsulation of transplanted tissue is an attractive approach to eliminate the risk of long-term immunosuppression and a method to overcome the immune barriers to islet xenoTx [42]. Encapsulated porcine islets are able to correct hyperglycemia in non-immunosuppressed rodents when transplanted subcutaneously [43]. However most studies demonstrate normalization of glycemia over a short period of time and long-term survival of encapsulated cells is still a concern because of fibroblast overgrowth around capsules [44].

Hepatocyte xenoTx is progressing rapidly, and recent experiments in small animal models used porcine hepatocytes transplanted into spleens of cirrhotic rats without immunosuppression and allowed restoration of metabolic functions and prolonged recipient survival [45]. These results demonstrate the feasibility of this approach to support liver failure by xenogeneic cells, but they need to be validated in large pre-clinical animal models.

Clinical xenotransplantation

Since the first attempt of clinical xenoTx in 1894 [2], that consisted to implant pieces of sheep pancreas subcutaneously to diabetic patients, some progress have been made. The first clinical cell xenoTx trial has been undertaken in Sweden [46] and consisted of porcine islet Tx into 10 diabetic patients. No improvement in recipient insulin requirement was observed, while demonstration of xenoislet cells survival was demonstrable in recipient with strong immunosuppression. In the last decade, the report of insulin independence for more than 9 months in one diabetic patient with Tx of encapsulated islet has enlightened a new approach for cell xenoTx [47]. It has confirmed that cell immunoisolation could protect graft from rejection while satisfying function could be achieved in human recipient.

Currently, clinical trials of neural cells xenoTx are ongoing, consisting of implantation of dopaminergic porcine

neural cells to treat basal neural ganglia for Parkinson's disease [48]. The present results are encouraging with demonstration of graft function and reduction in recipient drug requirement, presumably related to the privilege afforded by the blood-brain barrier in terms of rejection protection.

International skepticism has recently raised regarding data presented by Valdes *et al.* at the XIXth International congress of the Tx society in Miami in 2002 [49]. This group reported the achievement of insulin-independence in one of 12 non-immunosuppressed adolescents, and reduction of insulin-requirement in five, after Tx of porcine islet combined with porcine Sertoli cells. If these results confirm the feasibility of this approach, further experimental work in pre-clinical large animal models are necessary to confirm the validity of this approach [49].

Extracorporeal organ-support devices using xenogeneic cells

Metabolic liver support systems have been developed using xenogeneic hepatocytes [50–52]. Recently, an extracorporeal hybrid liver support system using primary porcine hepatocytes has been tested clinically in a phase I study in eight patients with acute liver failure, all of whom survived and were successfully bridged to liver alloTx [53]. Of importance, patients in contact with porcine cells through this extracorporeal bioreactor have not shown any evidence of porcine endogenous retrovirus (PERV) infection [54,55]. However, experience with extracorporeal liver support based on pig tissue did not demonstrate significant advantage over conventional intensive care therapy [50].

Xenozoonoses

A major issue concerning xenoTx is the potential transmission through the graft of animal derived pathogens to the human species. Xenozoonoses are one of the major arguments retaining clinical human xenoTx practice [56,57]. The recent example of SARS (severe acute respiratory distress) has clearly demonstrated the risk of xenogeneic viral pathogen transmission to human [58]. The history has shown the importance of this risk with the transfer of an influenza virus from pig to human in 1918, which led to millions of deaths worldwide [21,56]. Moreover, this concern will be amplified in case of Tx in which the recipient will be immunosuppressed. While the benefits of pig-to-human xenoTx are clear in terms of organ shortage, some potential risks remain. In particular, an inadvertent transmission of porcine microorganisms to the recipient is a concern, particularly if the recipient could transfer these microorganisms to other humans. Encouragingly, conventional barrier derivation technologies can remove most of the existing zoonotic microorganisms from donor herds and eliminate them as cause of concern. However PERV are unaffected by barrier derivation technologies, and the ability of certain PERV to infect human cells has been documented *in vitro* [59]. However, using the same *in vitro* conditions, it has not been possible to achieve viral transmission from cells from selected strains of miniature swine [59], and PERV transmission into humans or nonhuman primates has never been observed *in vivo* after exposure to living porcine tissues [54,55]. Recent reports have shown the absence of PERV transmission in immunosuppressed nonhuman primate models [59]. Studies reporting experience in human recipients of porcine tissues or in contact with porcine tissues (e.g. extracorporeal porcine liver bioreactor) have not shown any evidence of PERV transmission [54,55]. These discrepancies between *in vitro* and *in vivo* results could be explained by the presence of natural immunity against PERV in human and/or primates [60]. Two studies have recently demonstrated the presence of natural immunity against PERV in human serum. First, it has been shown that human serum can inhibit infectivity of PERV against human cells *in vitro* and moreover that human serum can promote viral inactivation through complement-mediated classical pathway [61]. This effect of human serum was inhibited by addition of synthetic Gal epitopes letting the authors conclude that Gal present on viral particles could have a role in this mechanism. A second study confirmed the previous results showing that human serum or anti-Gal antibody can inhibit human cell infection by PERV *in vivo* [in reconstituted severe combined immunodeficient (SCID) mice] [62]. These results emphasize the risk associated with the use of GT-KO pigs as donor for human xenoTx [63,64]. The use of tissue from such pigs could be associated with an increased risk of PERV transmission. When budding from cell membranes, PERV particles incorporate parts of the cell membrane. Thus, PERV particles from wild-type pigs express Gal epitopes and hence become the target for natural antibodies directed toward this epitope [62], while PERV particles from GT-KO pigs would not [64].

While porcine cytomegalovirus has been shown to be eradicated from piglets by early weaning from the mother, it has recently been shown to be associated with graft injury in pig to primate xenoTx model, and concerns about porcine cytomegalovirus have been raised as a potential transmissible pathogen in immunosuppressed xenotransplant recipients [65,66].

Ethics and regulation

Recently, a new and ongoing clinical trial of pig islet and Sertoli cell xenoTx in diabetic patients has been initiated

in Mexico City [49]. This trial has provoked some concerns in the scientific community, as it is not clear whether there is adequate oversight by a national regulatory authority in Mexico, and no results from pre-clinical animal experiments appear to have been reported in the scientific literature [49]. As several countries are performing or planning clinical xenoTx trials without specific regulations, individuals may freely travel to these countries to undergo such procedures. These 'xenotourists' will return home without monitoring after traveling and are at risk of developing or spreading new diseases.

The Ethics Committee of the International Xenotransplantation Association, a branch of the International Transplantation Society, has established guiding principles for new clinical trials of xenoTx [67,68]. The committee has emphasized the need for international cooperation to develop universally accepted oversight procedures and standards that would regulate the use of animal donors and monitor xenograft recipients. Among the International Xenotransplantation Association's suggested guidelines are the following:

1 Clinical xenoTx trials should be performed with oversight from a national governmental regulatory agency. Regulatory positions for the European Union have been reviewed recently by Tallacchini *et al.* [69].

2 Trials should be conducted with approval and oversight of an institutional panel to ensure the ethical conduct of human research, as well as ethical and humane treatment of non-human animals.

3 Trials should include the use of source animals housed in closed colonies from which known pathogens and potential pathogens have been excluded.

4 There should be adequate pre-clinical data to justify the clinical trial, with account being taken of the potential risk to the research subjects and to society.

In January 2004, the Executive Board of the World Health Organization (WHO) finalized resolution EB113.R5 on 'human organ and tissue transplantation' that includes guidelines on xenoTx. The text of this document is available in the six official languages of WHO at http://www.who.int/gb/, and was accepted by all member states of during the World Health Assembly in May 2004.

This report contains a draft resolution on xenoTx urging member states to follow specific guidelines, i.e. to allow xenoTx only when effective regulatory control and surveillance by national health authorities are in place; to draw up protective measures to prevent the potential secondary transmission of any xenogeneic pathogen that could have infected recipients of xenotransplants; and to support international collaboration for the prevention and surveillance of infections resulting from xenoTx.

As an international organization, the WHO could encourage the development of a cooperative international effort to develop such guidelines in collaboration with the International XenoTx Association. Further methods of monitoring that the WHO could encourage is the development an international registry of xenograft recipients that would allow collection and analysis of the results of all clinical trials. Trials should include long-term monitoring of the xenoTx recipients and their close contacts, and there should be a national repository where specimens from the organ-source pig and the human recipients are tested and stored.

Regarding the perception of xenoTx in the population, a Swedish survey based on the population and on patients awaiting for kidney Tx revealed that both groups favored cell rather than organ xenoTx. However, approximately 80% of the public and 90% of patients were in favor of continued research on xenoTx [70]. Recently an Italian survey based on university students showed that 78% of them approved the possibility of human xenoTx [71].

Conclusions

The use of xenogeneic tissues or organs in clinical trials could allow treatment of large numbers of humans in a near future. Several physiological, biological, immunological and infectious questions still need to be further evaluated and answered in experimental models (Fig. 1). Acute inflammatory and innate immune responses have to be

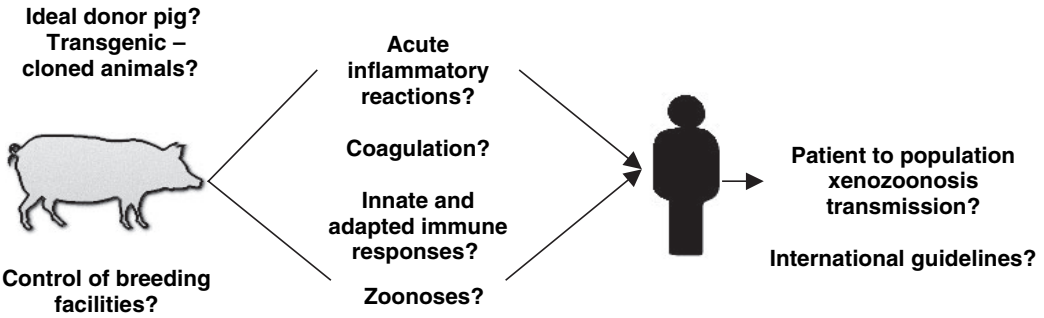

Figure 1 Current questions regarding pig-to-human xenotransplantation.

understood and prevented. Coagulation disturbances associated with xenogeneic endothelium or cells have to be overcome. Rejection of xenogeneic tissues is still a major hurdle and efficacy of tolerance induction protocols have to be developed in nonhuman primates. Finally, the risk of porcine xenozoonoses has to be evaluated.

Considerable progress has been made in experimental xenoTx in recent years, and clinical trials of xenogeneic cell transplantation are already underway. It has therefore become urgent to initiate international collaboration to develop a consensus on the necessary guidelines that will ensure future clinical trials are carried out ethically and safely.

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