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UPDATE: CARDIAC XENOTRANSPLANTATION

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Abstract

Purpose of review—To review the latest development in cardiac xenotransplantation in small and large animal models and related *in vitro* studies.

Recent findings—With the recent introduction of $\alpha 1,3$ -galactosyltransferase gene-knockout (GT-KO) pig organs for xenotransplantation, improved cardiac graft survival has been obtained. However, this experience has demonstrated the importance of pig antigens other than Gal $\alpha 1,3$ Gal (Gal) antigens (so-called nonGal antigens) as targets for primate anti-pig antibodies. Several *in vitro* studies have confirmed that, although the incidence and levels of anti-nonGal antibodies in non-human primates and humans are significantly less when compared with total anti-pig antibodies (i.e., anti-Gal + anti-nonGal), they can result in complement-mediated lysis of GT-KO pig cells. More recently, it has been demonstrated that regulatory T cells (Treg) suppress the cellular xenogeneic response, thus potentially preventing or reducing T cell-mediated rejection. The importance of thrombotic microangiopathy as a feature of the immune/inflammatory response and incompatibilities between the coagulation-anticoagulation systems of pig and primate are receiving increasing attention. Development of GT-KO pigs transgenic for one or more ‘anti-thrombotic’ genes, e.g., CD39 or tissue factor pathway inhibitor, may contribute to overcoming these problems.

Summary—Although GT-KO pigs have provided an advance over wild-type pigs as a source of Organs for transplantation into primates, further genetic modification of GT-KO pigs is required to overcome the remaining immune barriers before a clinical trial of cardiac xenotransplantation can be contemplated.

Keywords

$\alpha 1,3$ -galactosyltransferase gene-knockout; cardiac; heart; xenotransplantation

INTRODUCTION

Although innovative medical therapies, sometimes combined with support by a ventricular assist device, are effective in many patients with heart failure, heart allotransplantation (alloTx) remains the definitive therapy for end-stage heart failure. Despite its limitations for the patient, such as a long waiting time, often spent in a hospital intensive care unit, before a suitable human donor can be found, it remains the best treatment for end-stage heart failure.

Mulligan et al [1] have recently reported that the mortality of patients awaiting heart alloTx has declined over the past 10 years. The increasing use of ventricular assist devices may have

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contributed to the declining death rates. Although mechanical devices have proven valuable in the treatment of heart failure, the insertion of a foreign body is not ideal, as the device is susceptible to infection from episodes of bacteremia that occur during everyday life, and from other complications, such as thromboembolism. Infection of a ventricular assist device or total artificial heart remains a serious, often devastating complication because, if removal of a device is necessary, this threatens the life of the patient. Successful treatment of infection without device removal is difficult.

A readily available animal source of organs, tissues, and cells for clinical Tx (cross-species Tx or xenoTx) would resolve the increasing discrepancy between the availability of donated human organs and the demand for Tx. If pig organs could be transplanted successfully into human patients, the advantages would be numerous. The supply of organs would be unlimited, they would be available electively when needed, and the organ-source pig would be known to be free of specific microbes that might cause morbidity in the recipient.

The German Society for Thoracic and Cardiovascular Surgery [2] published its assessment of alternatives to heart alloTx. In view of the steadily improving results of heart Tx in the pig-to-nonhuman primate model [3,4], particularly of hearts from pigs homozygous for $\alpha 1,3$ -galactosyltransferase gene-knockout (GT-KO), where graft survival has reached almost 6 months, cardiac xenoTx is likely to be a valid option for the treatment of end-stage heart failure.

Although the introduction of genetically-modified pigs for xenoTx has increased the resistance of the organs to the xenoreactive immune response, there remain immunological and other barriers that currently prevent the clinical application of xenoTx.

IMMUNOLOGICAL ISSUES

Transplantation of an unmodified pig heart into a non-immunosuppressed (or standard pharmacologically-immunosuppressed) human or higher non-human primate results in destruction of the graft within minutes or hours by a process known as hyperacute rejection (HAR). In HAR, the recognition of pig antigens, predominantly Gal $\alpha 1,3$ Gal (Gal), by primate preformed (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 [5–7]. The relatively recent introduction of GT-KO pigs [8,9], that do not express the major antigenic target for primate anti-pig antibodies (Gal), has brought clinical xenoTx one step closer by avoiding HAR [3,4].

GT-KO hearts transplanted heterotopically into immunosuppressed baboons have survived for up to 6 months [3,4]. Graft failure was not from the typical features of humoral rejection caused by antibody-mediated complement activation, but from the development of a thrombotic microangiopathy that resulted in vascular occlusion and surrounding ischemic injury.

Anti-nonGal antibodies

These studies highlighted the remaining major immunologic problems that need to be overcome [10–12]. Although the GT-KO pig organs overcame the presence of anti-Gal antibodies in the nonhuman primates, and thus prevented HAR, there are clearly antibodies directed toward non-Gal targets that can result in early humoral rejection [13,14,15*,16]. The exact targets for these anti-non-Gal antibodies remain uncertain [17].

As the thrombotic microangiopathy seen in the GT-KO pig-to-baboon experiments [18] may, in part, be secondary to vascular endothelial cell activation from anti-non-Gal antibodies and complement, GT-KO pigs transgenic for one or more human complement-regulatory proteins

(CRP), such as CD46 (membrane cofactor protein, MCP) or CD55 (decay-accelerating factor, DAF), may inhibit the development of this complication [12].

Coagulation dysregulation

However, it is known that there are significant incompatibilities between the coagulation-anticoagulation systems of pig and primate that may predispose to the development of thrombosis in the transplanted pig vessels. This dysfunction might be overcome by the introduction into the pig of a human 'anticoagulant' or 'anti-thrombotic' gene [19*,20], such as tissue factor pathway inhibitor, hirudin [21,22], or CD39 [23].

Wu et al [24*] studied the coagulation cascade in the pathogenesis of HAR and in the early failure of pig hearts transgenic for a human CRP in the pig-to-baboon heterotopic heart Tx model. In this study, 14 of 27 hearts (11 hDAF, 3 hMCP) exhibited either HAR (n=10) or early graft failure (n=4). Dysregulated coagulation correlated closely with, and probably caused, primary graft failure.

Pigs with one or more of the above genetic modifications have been produced, but GT-KO pigs that express both a CRP and an anti-thrombotic gene are only just becoming available and have not yet been tested in *in vivo* models.

The need for genetically-modified pigs is recognized as being essential, as is the need for improved immunosuppressive regimens, possibly with the addition of anticoagulant or anti-thrombotic medication, such as acetylsalicylic acid [aspirin], clopidogrel bisulfate [plavix], enoxiparin sodium [lovenox], warfarin [coumadin], or heparin. Advances in immunosuppressive medication, such as co-stimulatory blockade, have allowed prevention of a T cell-dependent elicited antibody response [25,26,27*].

However, Byrne and colleagues have recently demonstrated that significant prolongation of xenograft survival after cardiac transplantation in a pig-to-nonhuman primate model can be achieved by improved immunosuppression, rather than through an increase in anticoagulation [28*]. In this study, high-dose immunosuppression was used in two groups where an improved median survival of 76 days was achieved in pig-to-baboon heterotopic cardiac transplants.

Heart perfusion models

Several recent efforts have been directed towards improving survival and function of cardiac xenografts. Brandl et al [29] have investigated the kinetics of anti-pig and anti-Gal IgM and IgG antibodies after perfusing human blood containing GAS914, a Gal trisaccharide conjugated to poly-L-lysine, through hDAF pig hearts using a working *ex vivo* model. They explored corresponding changes in parameters of heart function. When hDAF pig hearts were perfused with human blood containing GAS914, there was an immediate and extensive reduction in both anti-Gal IgM and IgG. Their study indicated that, by causing an immediate and profound reduction in Gal-specific antibodies, soluble Gal conjugates not only prolonged pig graft survival, but also improved the hemodynamic performance of the heart of hDAF pigs.

Charniot and colleagues [30] perfused seven small pig hearts with human blood using a Langendorff blood perfusion model. Reactive oxygen species were generated, probably promoting arrhythmias and impairment of left ventricular pressure. This group concluded that xenoTx was associated with a significant increase in ischemic injury and oxidative stress, factors that might play a role in the development of HAR.

Smolenski et al [31] perfused hearts from five hDAF transgenic pigs (generated by sperm-mediated gene transfer) *ex vivo* with human blood. The hearts were protected from HAR, were

relatively metabolically stable, and maintained mechanical function above the threshold level for life-support.

T regulatory cells

Porter et al [32] have recently studied CD4⁺ CD25⁺ regulatory T cells (Treg) in baboons to see whether Treg can modulate the xenogeneic immune response. The characterization of baboon Treg will be beneficial in experiments relating to tolerance induction. Treg were isolated from baboon lymph nodes, spleens, and blood. Porcine antigen-specific baboon CD4⁺ CD25^{high} cells were purified and expanded *in vitro*, and their effect on the baboon anti-pig xenogeneic response was studied. Baboon Treg suppressed the response to xenogeneic stimulation. This study suggests that adoptive transfer of expanded Treg into xenotransplant recipients may prevent cell-mediated rejection of grafts and potentially induce tolerance in the pig-to-baboon xenoTx preclinical model.

Accommodation

Accommodation occurs in ABO-incompatible organ alloTx and in rodent models of xenoTx, but has not yet been conclusively described in large animal models of xenoTx. In accommodation, a graft becomes resistant to destruction despite the presence of specific anti-donor antibodies and normal levels of complement [33–35]. An understanding of the mechanism of accommodation might provide clues to potential therapeutic manipulations of the donor or recipient that could improve the survival of xenografts.

Recently, Komori et al [36*] have shown that host accommodation could be more important than graft accommodation. The study was performed in a hamster-to-rat cardiac Tx model. Accommodated *grafts* expressing protective genes were rejected with an increase of both IgM and B-1 cells. However, in accommodated *hosts*, both IgM and B-1 cells decreased. Their results suggest that sufficient suppression of recipient B-1 cells, resulting in decreased titers of antibodies, may play an important role in the development of accommodation.

CELL TRANSPLANTATION AS A FORM OF XENOTRANSPLANTATION

Transplantation of isolated cells, such as myoblasts or stem cells, has been proposed for the repair of myocardial disease. The main hurdles in cell xenoTx are primary non-function, failure of neovascularization, microenvironment incompatibility, and cellular rejection. Small animal models are useful to study cell Tx especially in cardiac xenoTx. Recently, several studies were reported regarding survival of stem cells and myoblasts.

Dai et al [37] and Grinnemo et al [38] demonstrated survival and maturation of human embryonic stem cell (ESC)-derived cardiomyocytes in rat hearts. In the first paper, human ESCs survived several days during transportation between cities and for up to four weeks after Tx into hearts of nude rats, subjected to ischemia/reperfusion with minimal infarction damage. Human ESC transplanted into rat hearts continued to express cardiac muscle markers and exhibit sarcomeric structure, and were well-integrated into the endogenous myocardium. However, human ESC-derived cells did not escape immune surveillance in the xenograft setting in that they elicited a rejection response in immunocompetent rats.

Guo et al. [39] recently showed (in a model of myoblast-based cardiac repair) that allo-myoblasts survived better than xeno-myoblasts. Rat or human skeletal myoblasts were transplanted into infarcted rat hearts with or without immunosuppression. Immunosuppressive therapy enhanced the survival of skeletal myoblasts and improved heart performance.

Several studies in small animal models have also focused on prolonging xenograft survival. Ma et al [40] showed that adoptive transfer of double-negative Treg induced B cell death *in*

vivo and altered the rejection pattern of rat-to-mouse heart Tx, prolonging xenograft survival. They concluded that double-negative Treg may be invaluable in controlling B cell responses in xenoTx.

Zhen-Wei and co-workers [41] recently demonstrated that the expression of hemoxygenase-1, which was evaluated for its protective effect in TNF- α -induced apoptosis in human umbilical vein endothelial cells in the guinea pig-to-rat heart Tx model improved the survival of the xenograft by inhibiting inflammatory cell infiltration, degrading xenoreactive antibodies, down-regulating CD40L expression, and preventing apoptosis.

It has taken almost 20 years to progress from graft survival of a few minutes to survival extending over several months, and it took more than 10 years from the concept of genetically engineering pigs that do not express the Gal antigen before these pigs were developed and tested in non-human primates [27*]. Since the initial studies on the Tx of GT-KO pig organs in baboons in 2004, progress has been slow.

Surgical considerations

The majority of experimental studies have utilized the heterotopic (auxiliary) heart Tx approach. Because a heterotopic heart graft does not contribute to support of the circulation, orthotopically-placed grafts would be likely to fail earlier. Siepe et al [42*] investigated the anatomical differences between human and pig hearts that would require special care in cardiac xenoTx. They transplanted pig hearts into deceased human recipients. They drew attention to the following surgical points: 1) special care must be paid to the anastomosis of the donor-recipient pulmonary arteries because of different outflow angles; 2) the biatrial technique should be used rather than performing caval anastomoses as this avoids potential stenoses at the anastomoses of the SVC and IVC related to the differing angles at which these structures enter the heart; 3) the porcine left azygous vein that drains into the coronary sinus needs to be ligated to avoid bleeding. These technical considerations could not present any problems to trained cardiac surgeons.

Ricci et al [43] explored the value of right ventricular endomyocardial biopsy for the diagnosis of rejection after pig-to-baboon heterotopic cardiac xenoTx. Their results indicated that delayed xenograft rejection is a widespread process involving both right and left ventricles similarly. They concluded that histological assessment of right ventricular endomyocardial biopsy specimens is an effective method for monitoring acute humoral xenograft rejection after cardiac xenoTx.

Conclusion: Consideration of clinical trials

Increased consideration has been given towards clinical trials for potential candidates using pig organs or cells. Ibrahim et al considered the case for cardiac bridging as the first clinical trial of xenoTx [44]. The ideal adult patients might be those with severe biventricular dysfunction from ischemic cardiomyopathy with ventricular dysrhythmias, but who do not have any significant comorbidities, such as hemodynamic shock, hepato-renal failure with dysregulated coagulation, sepsis, or multi-organ failure.

An assessment of the experimental results that might justify the initiation of a clinical trial was made some years ago by the International Society for Heart and Lung Transplantation [45]. A prerequisite of a minimum of 60% graft survival for 3 months in a series of consecutive life-supporting (orthotopic) heart transplants in the pig-to-nonhuman primate model was considered necessary, with at least 10 recipients surviving for this period of time on a clinically-applicable immunosuppressive regimen.

With the GT-KO and newer genetically-modified pigs that are becoming available, and the advances in immunosuppressive agents that prevent an elicited antibody response, the immunological problems of xenoTx will steadily be reduced [46]. Furthermore, there is now less concern with regard to the transfer of porcine endogenous retroviruses to the recipient than previously [47,48]. ‘Physiological’ problems, however, particularly those relating to coagulation pathways, remain an important challenge that is being addressed aggressively by several groups. In this context, it is noteworthy that pigs transgenic for human TFPI and human CD39 have recently been produced and the use of organs from these engineered animals could result in improved survival of porcine xenografts transplanted into primates [48]. The prospect of pig organs resolving the critical shortage of human organs is getting closer. As the longest survival of a pig organ in a nonhuman primate to date has been the heart, cardiac xenoTx might provide the first clinical trial of solid organ xenoTx.

ABBREVIATIONS

CRP, complement-regulatory protein; DAF, decay-accelerating factor; ESC, embryonic stem cell; Gal, Gal α 1,3Gal; GT-KO, α 1,3-galactosyltransferase gene-knockout; HAR, hyperacute rejection; MCP, membrane cofactor protein; Treg, regulatory T cells; TFPI, tissue factor pathway inhibitor; Tx, transplantation.

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