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Current Progress in Xenogeneic Tolerance

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Abstract

Purpose of review—The present review updates the current status of research regarding the immunologic responses of the recipient following xenotransplantation. Additionally, we present the recent progress with attempts to induce xenogeneic tolerance induction.

Recent findings—There continues to be great interest in xenotransplantation. Recently, descriptions of the mechanisms responsible for attempted T cell xenogeneic tolerance in both large and small animal models have improved xenogeneic graft survivals. Additionally, the cellular signaling mechanisms, such as those involving CD39, CD44, and CD47 are proving to be highly important. Using the mixed chimerism approach to tolerance in xenogeneic model may be encouraging, especially given the recent clarification of the role for macrophage induced phagocytosis of xenogeneic donor cells.

Summary—Induction of tolerance to xenogeneic antigens has been accomplished only in small animals, however graft survivals in large animal models continue to improve. Further clarification of both the adaptive and innate immune responses to xenogeneic antigens are required for success to continue.

Keywords

Tolerance; Xenotransplantation; Thymus Transplantation; Mixed Chimerism; Pig-to-Nonhuman Primates

Introduction

Xenotransplantion is a rapidly changing field. In this review, we have focused on both the seminal publications that have driven research in xenotransplantation as well as the most recently published work and future endeavors. Here we discuss the importance of a tolerance strategy to xenotransplantation and the cellular responses of xenogeneic antigens.

1. Xenotransplantation remains at the forefront as a solution to the donor organ shortage

Because rates of end-stage organ disease are rising, the need for transplantable organs is increasing (1, 2). Unfortunately, there is vast discrepancy between the number of available

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donor organs and the number of patients on the waiting list (1,2). For these reasons, clinicians and scientists have searched for ways to expand the donor pool. One option is to regenerate organs or develop functional organs or cells *de novo*. Significant interest in tissue engineering has been generated by studies which have shown that allogeneic hepatocytes and islets of Langerhans can support the life of the recipients in animal models (3-6). In the case of hepatocytes, investigators have been successful in directing precursors toward the hepatic lineage using BMP-4 and b-FGF, however, function of the resultant tissue has been limited (3, 6). Recently, techniques for reprogramming adult tissues through gene manipulation in order to produce induced pluriopotent stem cells (iPS) have spawned interest in organ regeneration (7-9). Investigators were successful in developing foregut endoderm, which may potentially yield functional thyroid, thymic, and lung tissue, from human pluripotent stem cells by manipulating growth signals including TGF-beta (10). Along similar lines, when rat hearts were decellularized with detergents and reseeded with cardiac or endothelial cells, investigators were successful in producing a heart with about 2% of the function observed in an adult heart (11). While such technologies are innovative and may provide alternative sources of allogeneic organs in the future, these technologies have yet to yield life-supporting reprogrammed, de-novo, or regenerated solid organs in large animals.

Interspecies transplantation, or “Xenotransplantation,” offers the benefit of an inexhaustible supply of organs. Pigs are generally considered the best candidate for xenotransplantation donors (12,13) as they are physiologically and anatomically similar to humans. Because pigs constitutively express an antigen on their cell surfaces to which humans have pre-formed antibody, specifically alpha- 1,3-galactose, transplantation of swine grafts into non-human primates led to hyperacute rejection (14-16). In order to circumvent this problem, knock-out pigs that do not express the gene for this antigen were produced by nuclear cloning (17-19). Recent developments have shown that by using zinc-finger nucleases, geneticists can markedly increase the efficiency of eliminating the gal-antigen and that they can do so in a single transfection. These results have made the development of new lines of GalTKO swine far easier than previously thought (20). Utilizing GalT-KO pigs as donors, recent reports have demonstrated prolongation of life-supporting renal and orthotopic heart graft survivals of up 57 days (McGregor CGA. et al. IXA 2009 Venice. Early Cardiac Function and Gene Expression after Orthotopic Cardiac Xenotransplantation) and graft survivals of 179 days using hetrotopic heart models (21) have been realized.

2. Xenogeneic T cell responses and strategies to inhibit xenogeneic T cell Responses

The development of GalT-KO pigs overcame hyperacute rejection in non-human primates with low levels of cytotoxic non-Gal natural antibodies (22). However, the residual immunogenicity of these xenogeneic organs has prevented its adoption as a valuable clinical tool. In vitro assays assessing xenogeneic T cell responses between pigs and humans have indicated that xenogeneic cellular responses (both T cell proliferative responses and cell-mediated killing) are as strong or stronger than allogeneic responses (23-26). Allotransplantation of solid organs has been highly successful since the advent of immunosuppression. However, the immunosuppressive therapies that would be required to support the life of a xenotransplant recipient using immunosuppression alone would be highly toxic and possibly life-threatening.

Research designed to clarify the mechanisms by which T cell/antigen presentation (i.e. indirect vs direct) occurs in xenotransplantation has been dominated by small animal models. Early studies have shown that xenogeneic proliferation and lymphokine production are dependent on CD4+ helper T cells and self antigen-presenting cells (ie. the indirect

pathway) (23). Others have reported that mice which lack the ability to express MHC class-II on antigen presenting cells (APCs), but have normal T cell counts, showed markedly prolonged porcine skin graft survival, suggesting that xenogeneic T cell responses were dependent on the indirect pathway dependent (27). However, we and others have demonstrated that unlike the reported observations between mice and pigs, the direct pathway is strongly involved in the xenogeneic T cell responses between pigs and humans (24,25). Therefore, we believe that strategies directed at inhibition of the direct pathway must also be included for successful xenotransplantation between pigs and primates.

Co-stimulation blockade has been used in both large and small animal models of xenotransplantation with good results and may be useful for future tolerance strategies. Recent studies in pig to nonhuman primates have shown improvement in xenograft survivals using co-stimulation blocking with agents such as anti-CD40L, a monoclonal antibody (21,22,28-30). Because anti-CD40L, which has been shown to be effective, is no longer clinically available, alternative drugs are needed. Recent reports suggest that blocking T cell co-stimulation using inducible co-factor (ICOS), leads to increased graft xenograft survival in pig to mouse models (31,32). Survival of pig endothelial cells transfected with ICOS-Ig was prolonged in comparison to wild-type cells. Interestingly, the authors attributed this prolongation of the accumulation of CD4(+)CD25(+)Foxp3(+) cells. In addition, intra-graft levels of IL-10 were 2.8 times higher in the ICOS-Ig grafts when compared with wild-type (32). Large animal data have also shown compelling evidence that co-stimulatory blockade, specifically a CD40-specific monoclonal antibody, prolongs xenoislet survival (33). Neonatal islets have demonstrated better in-vivo function, however, the grafts eventually succumbed to rejection. On immunohistochemical analysis, the authors noted that the infiltrating cells were largely CD3+ and neither neutrophils nor CD20+ cells were present in peri-islet cellular infiltrates. This corroborates other studies demonstrating that the major cell types present in islet rejection are CD3+, which suggest us the efficacy of tolerance strategy (see next section).

Recently the effects of other antibodies, anti-CD44 and anti-CD39, on xenogeneic T cell responses have explored. It has been demostrated that the use of anti-CD44, which recognizes a cell adhesion protein (31), inhibited the T cell response (specifically the memory T cell response) to cardiac allografts (34). Based on these data, the same antibody was tested in a rat-to-mouse xenotransplant system. The authors found that without anti-CD44, skin graft survival was approximately 7 days, in comparison to more than 20 days with anti-CD44 treatment. This therapy was particularly effective in inhibition of the Th1 subtype (35). Robson and colleagues have defined and characterized the role of signaling by extracellular nucleotides and nucleoside derivatives in transplant rejection and have examined purinergic mechanisms of inflammation in transplantation (36). One of the key players in these processes is CD39, an ecto-nucleoside triphosphate diphosphohydrolase that hydrolyzes extracellular ATP and ADP to AMP and is uniquely expressed at high levels by vascular endothelium and also on T regulatory cells (Treg) (37-39). CD39 efficiently distinguishes resting and activated foxP3+ CD4+CD25+ Treg from other T cells in mouse and human/primate systems (40,41). Given species differences with low, intrinsic CD39 basal functional expression in porcine tissues (42), it might be predicted that transgenic pig cells (bone marrow, passenger leukocytes or vascular cells) over-expressing CD39 may exert immunosuppressive effects locally. Certainly, the cardiac grafts from CD39 transgenic mice are resistant to humoral-type rejection with elicited antibodies to Gal (43) as well as effects on T cells.

3. Current Strategies of T cell Tolerance in Pig-to-Baboon Solid organ Transplantation

When GalTKO pigs became available for xenotransplantation in 2003, we began GalTKO pig-to-baboon life supporting xenotransplantation. Despite the use of comprehensive immunosuppressive regimens including anti-CD40L mAb, GalT-KO kidneys were rejected by day 34 (22). Subsequent studies by other groups similarly demonstrated graft survivals less than 4 weeks (44)(Cozzi E et al., and Brancho G et al. 2009 IXA congress). Histologically, and supportive of our in vitro data between pig and human, acute cellular xenograft rejection (ACXR) was observed in these failed GalT-KO kidney grafts (45: Shimizu A et al. JASN 2011 in press). In animals treated with immunosuppression protocols alone, the number of graft infiltrating cells was observed to increase markedly after transplantation. These cells were mainly CD3+ T cells and CD68+ macrophages, as well as small numbers of CD20+ B cells. Two-color immunohistochemistry identified the presence of TIA-1+ cytotoxic granules in many infiltrating CD3+ T cells, indicating that these T cells were cytotoxic. Furthermore, CD3+ T cells infiltrated the tubules, proximal tubular cells, glomerular capillaries, and underneath the endothelial cells of small arteries. These findings were associated with tubulitis, capillaritis, acute glomerulitis, and endothelialitis. In contrast, grafts transplanted using our immunotolerance protocol, demonstrated fewer interstitial mononuclear cells and no or little tubulitis, capillaritis, glomerulitis, or endothelialitis (45: Shimizu A et al. JASN 2011 in press)

For these reasons, adoption of a tolerance approach to inhibit xenogeneic T cell responses in a specific manner is of crucial importance, and our preliminary data utilizing vascularzied donor thymic grafts co-transplanted with kidneys from GalTKO pigs in non-human primates have been encouraging (22,46).

Early studies in mice demonstrated that transplantation of fetal or neonatal pig thymic tissue to thymectomized mice produced tolerance to pig skin grafts (47,48). Further studies proved that polyclonal, functional human T cells can develop in swine thymic tissue and these cells exhibit donor specific unresponsiveness (49). Studies in the authors' lab have shown the advantage of using pre-vascularized thymic tissue over non-vascularized tissue. During the period of neovascularization, thymic graft architecture is lost due to ischemic injury (50) which inhibits the thymic deletion of xenoreactive recipient T cells. Thus, we have developed two methods of transplanting vascularized thymic tissue; either by direct vascular anastomosis of the thymic blood supply (51), or as a thymokidney (prepared by injecting autologous thymic tissue under the renal capsule and allowing two months for neovascularization prior to transplantation) (52). Our studies have demonstrated functional thymopoesis in transplanted thymic grafts and donor specific tolerance induction across a full MHC mismatch barrier in an allogeneic miniature swine model (53-55). By utilizing GalT-KO animals as donors for either vascularized thymic tissue or thymokidneys, our initial attempts have resulted in markedly prolonged renal xenograft survival with normal renal function for greater than 80 days (22). Because infection was a concern in the initial group, a modified immunotolerance regimen was designed without steroids and whole body irradiation. The modified regimen led to improvement in average survival to greater than 50 days from 34 days (46). Long-term survivors demonstrated donor specific unresponsiveness in vitro in CTL assays along with evidence of thymopoiesis in transplanted porcine thymic grafts, suggesting these baboons were on a path toward on tolerance.

Recent findings in this vascularized thymus plus kidney model have shown the importance of optimal T cell depletion in the induction period. We have determined that T cell depletion 1) was essential for avoidance of rejection, 2) may lead to lethal infection if too extensive, 3) likely requires maintenance at an optimal level (in our experience between 50-150 T cells/ul

in the peripheral blood) during the first two weeks following induction of xenotransplantation tolerance of vascularized grafts (56)

4. B-cell responses and B cell Tolerance Strategies across Xenogeneic Barriers

T cell responses may not be the sole cause of xenograft rejection. A number of groups have investigated the role of B cells and plasma cells, and more recently focus has been directed at innate immunity in xenograft rejection.

Mixed chimerism is achieved by the transplantation and engraftment of bone marrow or blood progenitor cells such that donor and recipient hematopoietic cells coexist in the recipient. When this occurs, the recipient becomes tolerant of donor antigens. This is thought to occur by a central mechanism in which donor cells migrate to the thymus where they induce clonal deletion of maturing donor-reactive thymocytes. This process also leads to B cell tolerance in bone marrow (57).

Studies have shown that this mechanism is active across a xenogeneic barrier. In fact, mixed chimerism has resulted in long-term survival of rat cardiac grafts in mice, a concordant species barrier which lacks xenogeneic natural antibodies (58). More recently, in order to mimic discordant barriers using rodents, investigators used GalTKO mice as recipients and showed that successful engraftment of GalT+/+ bone marrow leads to disappearance of anti-Gal antibodies. Additionally, these animals successfully accepted donor-matched heart grafts for > 100 days, indicating the presence of T cell and B cell tolerance to GALalpha1,3GAL-expressing heart xenografts (59). Because humans may carry a titer of anti-pig antibodies much higher than that of the mice model, a second similar experiment was conducted by this group using mice sensitized with Gal antigens. Although the standard model of non-myeloablative conditioning and bone marrow transplantation did not lead to engraftment, authors found that larger doses of bone marrow did result in engraftment and once again, lasting B cell tolerance. These mice subsequently accepted cardiac xenografts without signs of rejection (60). The data from these rodent models suggested that a non-myeloablative induction regimen with subsequent bone marrow transplantation leading to mixed chimerism may be capable of tolerizing porcine antigens-reactive B/plasma cells.

Despite the promising results in the rodent models, xeno-bone marrow transplantation in pig-to-non-human primates has, thus far, not been successful. Initial attempts to induce mixed chimerism in a clinically relevant model involved transplantation of pig bone marrow (BM) cells into cynomolgus monkeys and baboons. These animals failed to demonstrate macrochimerism as determined by flow cytometric analysis (FACS), but did have intermittent detection of pig cells by PCR (61-63). One hypothesis proposed to account for this finding was that porcine progenitor cells which were Gal negative had engrafted but mature cells, which would express Gal, were rapidly destroyed preventing detection by FACS. Subsequent studies have been carried out to test ability of bone marrow taken from the GalT-KO miniature swine to engraft in a baboon treated with a non-myeloablative conditioning regimen (64). Although in vitro results, specifically mixed lymphocyte reaction (MLR) data, demonstrated non-specific hyporesponsiveness, most of porcine cells were cleared within 24-48 hours post-bone marrow infusion (65), indicating that additional strategies are required to achieve persistence of donor chimerism in pig to primate models.

4. Innate response

Recent work to circumvent this rapid clearance of BM cells has focused on innate immunity and macrophage-associated mechanisms. Specifically, attention has focused on SIRP-alpha,

a transmembrane protein with intracellular tyrosine kinase activity that is present on macrophages, dendritic cells, and neutrophils. The ligand of this receptor is CD47, and recognition of CD47 by SIRP-alpha down-regulates phagocytosis by macrophages (66). Thus, if a donor cell does not express a form of CD47 which can be recognized by the recipient, the cell is at risk for phagocytosis (67,68). In a small animal study in which human hematopoietic stem cells were transplanted into severely immunocompromised mice to allow for human hematopoiesis, the authors found significant improvement in hematopoiesis and engraftment in mice that were transgenic for human SIRP-alpha (69). Specifically, the mice transgenic for hSIRP-alpha had higher levels of CD45+ lymphocytes in the blood than controls.

In a related model, investigators produced mice in which were T and B cell deficient but that expressed a SIRP alpha which recognized human CD47. Thereafter, control B cells and B cells that expressed human CD47 (both taken from a lymophoma cell line) were xenotransplanted. Post-transplantation, only the CD47 cells survived, implying a survival advantage to CD47 expression. Interestingly, authors then macrophage-depleted the recipients and found that both populations survived, highlighting the importance of the interaction between CD47 and SIRP-alpha and the downstream effects of macrophage induced phagocytosis (70). Because it appears that the innate immunologic responses, specifically macrophages, are associated with xenogeneic rejection, the use of human-CD47 transfected bone-marrow holds significant potential. If pig bone marrow or thymic precursors could be successfully transfected with human-CD47 and thus protected from macrophage induced phagocytosis, this may provide a favorable environment for mixed chimerism leading to donor-specific unresponsiveness.

Conclusions

Taken together, both in vivo and in vitro studies indicate that xenogeneic T cell responses play a major role in cellular rejection. Inhibition of these responses is crucial because even a small number of xenoreactive T cells can potentially initiate other cellular responses. Induction of donor specific T cell tolerance across xenogeneic barriers is not only essential to the success of xenotransplantation but is also an attainable goal. In the next several years, tolerance strategies combined with genetic manipulation of donors through transgenic or knockout technologies could make xenogeneic organs more acceptable to patients in need of transplants.

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Key points

1. Xenotransplantation remains the most viable option for large scale expansion of the donor organ pool.
2. T cell responses to xenogeneic tissues were initially underappreciated, but are now thought to represent one of major contributors to the process of xenogeneic rejection
3. Understanding the role of the thymus in xenogeneic responses is crucial for improvement in long-term outcomes
4. Chimerism may provide an opportunity to establish xenogeneic tolerance