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Achieving tolerance in pig-to-primate xenotransplantation: Reality or fantasy

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Abstract

Because the immunologic differences between species are far greater than those within species, it is likely that the amount of immunosuppression that would be required for successful xenografting would be so much greater than that now used for allografting, that the side-effects and complications would be unacceptable. Tolerance approaches to xenotransplantation would overcome this concern. Studies in humanized mouse models have demonstrated that human T cells can be tolerized to porcine xenografts, providing important proofs of principle of the potential feasibility of pig-to-primate xenograft tolerance. The results available from studies of pig-to-primate xenotransplantation to date have demonstrated that while chronic immunosuppressive drugs have not completely avoided either T cell responses or humoral rejection, approaches directed toward tolerance induction have been encouraging with regard to avoiding immunization at both of these levels.

Keywords

Xenotransplantation; Clinical xenografting; Transplantation; Tolerance; Chimerism

1. Introduction

In the first issue of the journal Xenotransplantation in 1994, it was predicted in the Editor's preface that “the success of clinical xenografting will depend, at least in part, on finding ways of inducing tolerance across xenogeneic barriers rather than relying entirely on non-specific immunosuppressive agents” [1]. As is clear from all of the contributions to the present special issue of Transplant Immunology, neither tolerance nor success of clinical xenotransplantation has yet been realized in the intervening 14 years. However, in the opinion of the authors of this article, the same prediction is still valid, and considerable progress has been made toward achieving the kind of tolerance on which clinical xenografting will depend. It is our intent here to summarize the results of pig-to-primate organ transplantation to date that support the need for tolerance, to present evidence from a humanized mouse model that tolerance of the human immune system to transplanted pig tissues is possible, and to present the current status of studies designed to induce tolerance to pig organs in primates.

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2. Current status of pig-to-primate organ xenotransplantation using chronic immunosuppression

Efforts to avoid humoral rejection have included the use of transgenic donors expressing complement-inhibitory proteins such as hDAF [2], CD59 (membrane attack complex inhibitor) [3], and hMCP (human membrane cofactor protein, CD46) [4], as well as the elimination of Gal expression on pig cells. Despite the success of these efforts to control hyperacute rejection (HAR), severe delayed xenograft rejection (DXR) and acute cellular rejection (ACR) have not been avoided [5,6].

Life-supporting kidney transplantation with intensive chronic immunosuppressive therapy, using hDAF pigs in a pig-to-baboon model, have achieved survivals between one and two months [7]. The largest study of hDAF kidney transplants into baboons using chronic immunosuppression was reported by Garcia and Zhong et al. in 2004 [7,8], with hDAF kidneys sustaining life from 4 to 75 days, with a mean survival of 20.7 days. Similarly, we have achieved hDAF renal survival of up to 29 days [9] using chronic immunosuppression, and achieved only minimal improvement using GalT-KO kidneys with a similar protocol [10]. Zhong's group has reported maximum survivals of only 16 days [11] using GalT-KO kidneys. Despite the use of immunosuppression, including partial T cell depletion along with other immunosuppressive drugs, the rejected GalT-KO kidneys in these studies showed severe cellular and humoral rejection, along with bright staining for IgG in glomeruli and peritubular capillaries. Anti-pig antibodies in the circulation also increased significantly following transplantation [9,11], indicating that the eradication of cell surface α-Gal epitopes did not preclude the induction of a T cell-mediated response against the xenograft.

Heterotopically transplanted pig hearts survived longer than life-supporting kidneys in both hDAF and GalT-KO models, having less mononuclear cellular infiltrates in grafts. However, the likelihood of survival of these heterotopic hearts was undoubtedly increased by the fact that they did not have to support life of the recipients. McGregor et al. reported survival of heterotopic hCD46 hearts between 56 and 113 days [12], but survival of orthotopically transplanted hearts was limited to 8 weeks (8th IXA Congress 2005, Göteborg, Sweden, Abstract #O3:05), which is the longest reported survival for a life-supporting xenogeneic heart transplantation in a pig-to-baboon model. Although one animal in our center maintained a GalT-KO heterotopic heart graft for 179 days [13], this also was not markedly greater than the maximum of 139 days for an hDAF graft [14]. The presence of less cellular infiltrate in xenogeneic hearts has led some investigators to suggest that xenogeneic T cell responses are not a major cause of heart graft rejection, particularly in the heterotopic model [15]. However, the Mayo group has reported extended survival of CD46 transgenic hearts resulting from increased T cell immunosuppression with high-dose tacrolimus and sirolimus, suggesting that T cell responses do play an important role in heart graft rejection [16]. In addition, histology of rejected hearts in our center revealed thromboic microangiopathy, with deposition of anti-pig IgG, anti-pig IgM and C4d, along with a small amount of T cell infiltrates [17].

The results of these studies suggest that even small numbers of T cells can initiate a cascade of antibody and other cellular immune responses [18]. These findings are consistent with those of Korsgren and colleagues, who demonstrated that even very small numbers of T cells were sufficient to initiate rejection of porcine islets by macrophages in T cell deficient rodents [19,20]. We provided additional evidence for the role of T cell responses in xeno heart graft rejection when we reported that (1) xenogeneic T cell responses between humans and pigs are at least as strong as, or stronger, than allogeneic T cell responses [21], and (2) development of anti-Gal antibodies is to some degree T cell dependent [22]. In addition, our recent data indicate that T cell responses are also essential for macrophage infiltrates in response to pig islets in a humanized mouse model [23]. Consistently, the survival of porcine islet xenografts was limited

in heavily immunosuppressed baboons receiving porcine islet transplants by T cell-mediated rejection that was associated with cytokine responses to the donor [24]. In summary, it appears that inhibition of xenogeneic T cell responses is essential to the survival of islet, kidney and heart xenografts.

The level of immunosuppression needed to control these T cell responses and prolong xenograft survival has been prohibitive and associated with significant morbidity and mortality. Additionally, these regimens have not yielded survival times that would merit transition to clinical trials. Given these factors, we believe that the induction of tolerance will be a necessary part of the successful treatment regimen for clinical xenotransplantation.

3. Studies in humanized mice

Two successful approaches in large animal models toward the induction of tolerance of organ transplants across major histocompatibiltiy barriers have been the use of mixed hematopoietic chimerism [25] and of vascularized thymus transplantation [26]. That such tolerance is possible in humans has recently been demonstrated by the successful clinical application of the mixed chimerism approach to renal allografts [27]. Both of these approaches have now been shown to be capable of tolerizing human T cells to porcine xenografts in humanized mouse models, providing important proofs of principle of the potential feasibility of pig-to-primate xenograft tolerance.

3.1. Mixed xenogeneic chimerism induces human T cell tolerance to xenogeneic porcine donors

The demonstration that mixed chimerism can tolerize human lymphocytes has been made possible by the development of mice that are transgenic for the porcine hematopoietic cytokines IL-3, stem cell factor (SCF) and GM-CSF [28]. In contrast to wild-type mice, immunodeficient pig cytokine-transgenic mice allowed durable engraftment and hematopoietic function of porcine hematopoietic stem cells to occur. Importantly, the recipient thymus was shown to be seeded with porcine APC's, suggesting that tolerance of xenogeneic T cells might be achieved with this approach [29]. Such tolerance was first demonstrated by transplanting murine hematopoietic stem cells into pig cytokine-transgenic, immunodeficient mice with established porcine hematopoietic chimerism, which allowed the development of murine T cells that were tolerant to the pig, as demonstrated by the specific absence of mixed lymphocyte responses to the donor pig, a lack of antibody response to the donor, and donor-specific skin graft tolerance [30].

This approach was next extended to the human-pig combination by allowing human T cells to develop in immunodeficient porcine-cytokine-transgenic mice. Robust human T cell reconstitution was achieved via implantation of human fetal thymus and liver tissue under the kidney capsule and i.v. injection of CD34 + cells from the same fetal liver. Such animals developed human immune systems, including T cell, B cell and APC repopulation, restoration of lymphoid organ structure, the capacity for graft rejection and both proliferative and class-switched antibody responses following immunization [23,31,32]. Porcine class II + cells were detected in the human thymi of animals that had also received porcine bone marrow, and the human T cells that populated the periphery were shown to be tolerant of the donor pig. Tolerance was demonstrated by the long-term coexistence of human and pig chimerism, in MLR reactions and, importantly, by donor-specific skin graft acceptance [33].

All of the above studies relied on the use of NOD-SCID immunodeficient mice, while efforts to achieve durable porcine chimerism in immunocompetent mice and non-human primates were unsuccessful. We are therefore exploring additional barriers to xenogeneic hematopoiesis. These barriers have been shown to include the species-specificity of certain

hematopoietic cytokines [29] and adhesion molecules [34–38], as well as several components of the innate immune system, including natural antibodies [39], antibody-independent complement activation [40], NK cells [41] and macrophage-mediated destruction of xenogeneic hematopoietic cells [42]. Many of these barriers may potentially be overcome by combining genetic modifications of the source animals, including the existing GalT-KO swine and pigs transgenic for human complement regulatory proteins. NK cell-mediated resistance might likewise be overcome by introducing human class I genes into the pig and generation of a human CD47 transgenic pig might go far in overcoming the macrophage-mediated elimination of xenogeneic hematopoietic cells [43], since introduction of this human receptor has been shown to reduce markedly the phagocytosis of porcine cells by human macrophages [44]. Two further advantages of this approach include the ability of mixed chimerism to tolerize natural antibody-producing B cells for all specificities expressed by the donor [45] and the ability of mixed xenogeneic chimerism to tolerize NK cells [46]. Thus, with further studies, it remains likely that this mixed chimerism approach will also be applicable to pig-to-primate xenotransplantation.

3.2. Thymic transplantation induces T cell tolerance to highly disparate xenografts

The thymic transplantation approach was first shown to induce robust xenograft tolerance in a pig-to-mouse transplantation model, in which murine recipients of porcine thymic grafts demonstrated donor-specific unresponsiveness in mixed lymphocyte reaction (MLR) assays and specifically accepted skin grafts from the porcine thymic donors [47,48]. Normal, immunocompetent mice that were thymectomized (ATX), then treated with T cell depleting mAbs, permitted engraftment, growth and function of fetal porcine (FP) thymus (THY) tissue grafted under the recipient kidney capsule [47]. The pig THY replaced the host THY, permitting peripheral T cell reconstitution [47,49,50] and donor-specific xenograft tolerance was achieved [47,51]. Murine T cells entering the periphery were functional and cleared opportunistic infections [48]. Both porcine and murine APC present in FP THY grafts mediated intrathymic negative selection [47,49,51,52], while positive selection was mediated exclusively by porcine donor MHC [49,53,54].

Perhaps most relevant to the subject at hand, studies using a humanized mouse model have documented the feasibility of this approach for tolerization of human T cells to pig. Porcine thymus grafts were shown to support human T cell development from stem cells [55]. These human T cells developing in porcine thymus grafts were functional but were specifically unresponsive to the MHC of the donor pig in MLR assay, while responding normally to human alloantigens and porcine xenoantigens MHC-mismatched to the donor. The repertoire of human T cells developing in these porcine xenografts was both diverse and normal [56]. Thus, porcine thymus grafting has been shown to be capable of supporting normal human T cell reconstitution with specific tolerance to the porcine source animal.

4. Current status of tolerance induction in primates

The same two approaches have been studied for the induction of T cell tolerance to pig organs in baboons. Although full tolerance has yet to be achieved, there has been considerable progress in both approaches.

4.1. Mixed chimerism

Using either miniature swine or hDAF pigs as donors, the source of hematopoietic stem cells (HSC) infused into irradiated baboons has been either bone marrow (BM) [57,58] or cytokine-mobilized peripheral blood progenitor cells (PBPC) [59,60]. In the initial studies, administration of $2–3 \times 10^8$ pig bone marrow cells/kg along with swine recombinant growth factors pIL-3 and pSCF to immunosuppressed baboons led to transient and low levels of

chimerism as detected by FACS analysis, although porcine CFU were detectable in the recipients for over 6 months by PCR [57,61,62]. Further indication that pig stem cells were probably present at very low levels was the fact that sequential assays were variably positive over time [57].

Subsequent studies attempted to increase engraftment by raising the dose of progenitor cells administered through the use of cytokine-mobilized PBPC, achieving doses as high as 2–4 × 10^{10} cells/kg following mobilization and pheresis [62,63]. Even after administration of these enormous doses of pig cells, they were generally detected in the baboon circulation for only 2–5 days, although one animal demonstrated a second appearance of pig cells in the circulation on days 16–21 [64]. Specific hyporesponsiveness at the T cell level was observed in these animals by in vitro assays, suggesting a functional effect of the transient engraftment. It was reasoned that the failure of engrafted swine HSC to achieve peripheral chimerism might be due to the appearance of Gal on progeny of these cells and elimination by natural anti-Gal antibodies (Nab). If so, use of HSC from the new GalT-KO swine to baboons may achieve higher levels and longer duration of peripheral chimerism, and studies toward this goal are in progress. Preliminary data appear to confirm this possibility ([60] and unpublished data). In particular, two baboons treated with a non-myeloablative preparative regimen and receiving large doses (1–2 × 10^9 cells/kg) of GalT-KO bone marrow showed evidence of pig progenitor cell engraftment up to day 28, and did not develop antibodies to GalT-KO cells, despite making antibodies to two antibody reagents to which they had been exposed (manuscript in preparation). We consider failure to be immunized to pig antigens in these otherwise immunocompetent animals to be an indication that tolerance of pig antigens had been induced by the engrafted GalT-KO bone marrow cells.

4.2. Thymic transplantation

Yamada and colleagues have transplanted primarily vascularized thymic tissue from swine to baboons, following T cell depletion and thymectomy of the recipients, in an attempt to induce tolerance at the T cell level [10,65]. The thymic tissue was either in the form of “thymokidneys” – i.e. donor kidneys in which autologous thymic tissue had been allowed to engraft for 1–2 months under the kidney capsule before use as a renal transplant [66]; or vascularized thymic lobes [67]. As for mixed chimerism studies, the initial attempts were carried out using miniature swine or hDAF swine as donors. The vascularized xenografts survived for up to 30 days, with evidence of viable thymic epithelium and Hassall's corpuscles in the thymic implants, and with evidence of donor-specific hyporesponsiveness by in vitro assays in some of the animals after immunosuppression had been stopped. However, all grafts were rejected, coincident with the return of anti-Gal antibodies. In addition, the rejected grafts showed evidence for humoral rather than cellular rejection. It was therefore concluded that vascularized thymic tissue grafts induced specific T cell hyporesponsiveness, with the consequent absence of new, T cell-dependent antibody responses. Again, recurrence of anti-Gal antibody appeared to be resistant to down-regulation, and there was no evidence for accommodation.

In order to avoid the effects of these resistant and recurrent anti-Gal responses, the most recent studies of this modality have utilized GalT-KO pigs as donors of vascularized thymic tissue and kidneys. This change appears to have achieved a major increase in survival of renal xenografts compared to the previous studies using the same protocol [10,68]. Thus, in contrast to results with chronic immunosuppression (see above), co-transplantation of vascularized thymic tissue along with GalT-KO kidneys in an attempt to induce tolerance led to prolonged survival of functioning kidney xenografts, without the development of anti-donor T cell responses or of induced antibody, and with maintenance of third-party alloresponses [10,68]. This success is reflective of previously reported tolerance induction with allogeneic thymus transplantation [26,69–71], and of tolerance approaches in the pig-to-mouse and pig-to-

humanized mouse studies described above. Although the initial regimen utilized for GalT-KO porcine thymus-kidney transplantation in baboons led to frequent infectious complications, the maximum survival of life-supporting kidneys was prolonged from a previous record of about 30 days to over 80 days. Most importantly, the longest surviving kidneys were still functioning and showed relatively normal gross appearance and histology right up to the time that the animals expired or were sacrificed for other reasons.

5. The future of xenograft tolerance

Based on the studies discussed above, we are encouraged that successful induction of T cell tolerance across the pig to human xenogeneic barrier is not only achievable, but has already been achieved in humanized mice. Based on histologic and in vitro evidence, tolerance may also have been achieved in the pig-to-primate model, although titration of immunosuppression has not yet been complete in any primate recipient, which would be the most relevant in vivo criterion of tolerance. The studies described above using the mixed chimerism approach are encouraging with regard to tolerizing to avoid a humoral response, while the vascularized thymic approach showed evidence for tolerization of the cellular response as measured by specific non-responsiveness in CML. There is reason to hope that a combination of these approaches could lead to tolerance at both the T and B cell levels, bringing the success of xenotransplantation closer to clinical applications.

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