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XENOTRANSPLANTATION AND TOLERANCE

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1. ABSTRACT

Donor organ availability has become a major limiting factor in the progress of allotransplantation. This, and advances in genetic engineering in pigs, have led to increasing interest in the use of xenogeneic organs. In view of the greater difficulty encountered in overcoming immune responses to xenografts than to allografts, the success of clinical xenotransplantation may depend on finding ways of inducing specific hyporesponsiveness, or tolerance, across xenogeneic barriers rather than by relying on nonspecific immunosuppressive agents. This review discusses the barriers to xenogeneic organ transplantation and the approaches that are being developed to overcome them, with the emphasis on methods that attempt to induce tolerance.

2. INTRODUCTION

In view of the greater difficulty encountered in overcoming immune responses to xenografts than to allografts, it is likely that both acute and chronic rejection will be major obstacles to xenogeneic organ transplantation (Tx) even if natural antibody-induced rejection can be overcome. For this reason, the success of clinical xenotransplantation (XTx) may depend on finding ways of inducing specific hyporesponsiveness, or tolerance, across xenogeneic barriers rather than by relying on nonspecific immunosuppressive agents that are accompanied by risks of opportunistic infection, malignancy and drug toxicity.

Tolerance would obviate the need for chronic immunosuppressive therapy.

In this review, we shall briefly discuss the barriers to xenogeneic organ transplantation and the approaches that are being developed to overcome them, with the emphasis on methods that attempt to induce tolerance.

3. DEFINITIONS

Xenotransplantation (XTx) refers to the transplantation of tissues or organs between different species. When the human is considered as the potential organ recipient, XTx includes nonhuman primate-to-human or nonprimate mammal-to-human transplantation. There have been clinical attempts in both of these models, i.e. both nonhuman primates and other mammals, such as pigs and sheep, have been used clinically as sources of organs (1). In experimental settings, many different models have been described (2).

As originally proposed by Calne, XTx can be classified as either *concordant* or *discordant* (3). When *hyperacute rejection* occurs (HAR, occurring within minutes or hours), the donor-recipient pair is considered *discordant*. If rejection is delayed longer than 24 hours and follows a pattern similar, but possibly accelerated, to allograft rejection, he has suggested that the donor-recipient pair be considered *concordant*.

The major differences between these two groups is that in discordant pairs, HAR results from the existence of preformed natural antibodies in the recipient directed against antigens on the donor species vascular endothelium. These antibodies bind to the antigens on the vascular endothelium and activate complement, resulting in HAR. Because these antibodies are produced in the host without the need for prior immunization by a specific organ transplant, they are referred to as *natural (or preformed) xenoreactive antibodies*. When transplantation is carried out between concordant species, there is no or a very low level of natural xenoreactive antibody in the recipient directed at donor antigens. However, a rapid induced response develops, leading to rejection more rapidly than would occur in allograft models.

The pig is widely believed to be the most suitable donor for XTx into humans (4). In our own laboratories, we have focused on the use of partially-inbred miniature swine as a potential organ source. These MHC-inbred miniature swine have been produced by a selective breeding program over the last 25 years, and have a variety of advantages as potential sources of xenogeneic organs. These include anatomic similarity of the organs to humans, including size, and genetic characterization for MHC.

From the immunological point of view, the pig is discordant to humans. This is based on the presence of Galα1-3Gal (Gal) on the vascular endothelium of the pig and of anti-Gal antibodies in humans. All placental mammals, except humans, apes, and Old World monkeys, express a functional α-galactosyltransferase (αGT) gene, resulting in the expression of Gal epitopes on many tissues, including the vascular endothelium (5). Because animals that express a functional αGT gene are immunologically tolerant to Gal, they do not produce antibodies that bind the Gal epitope. In contrast, humans, apes, and Old World monkeys carry a nonfunctional αGT gene, the function of which appears to have been lost during evolution approximately 30 million years ago (6), and they develop antibodies to this oligosaccharide during infancy (7).

4. PATHOGENESIS OF XENOGRAFT REJECTION

4.1. Hyperacute Rejection (HAR)

The initial barrier to Tx of a pig organ into a primate is HAR, which results in graft loss within minutes or hours. This process is initiated by the binding of natural xenoreactive antibodies against discordant antigens on the vascular endothelium of the donor organ, which results in activation of the classical pathway of complement. In some species combinations, the alternative pathway of complement activation is believed to play a role, and evidence has been put forward to suggest that in humans this can occur from dimeric IgA binding to the pig endothelium (8). However, the classical pathway is considered more important.

Macroscopically, the transplanted organ swells (sometimes to twice its normal size and weight), becomes black from a combination of interstitial hemorrhage and ischemia, and ceases functioning. Histopathologically, HAR is characterized by disruption of the vascular endothelium, interstitial hemorrhage, edema and thrombus formation, which is initially more accentuated in the venous system than in the arterial. In some tissue and species combinations, there is marked infiltration of polymorphonuclear cells. Immunohistologically, HAR is characterized by IgM, IgG, IgA and complement deposition on the vascular endothelium.

4.2. Acute Humoral Xenograft Rejection (AHXR)

When HAR is prevented (by methods that are described below), AHXR develops within days or weeks and, at present, remains the major immunological barrier to successful XTx. Macroscopically, AHXR appears as a patchy discoloration on the graft, which reflects small areas of congestion and/or focal ischemia. Hemorrhagic spots may develop, indicating interstitial blood extravasation. There are as yet no clinical laboratory data that are diagnostic of the development of AHXR, although it can be associated with a progressive thrombocytopenia, due to consumption of platelets in the graft, and/or disturbance of coagulation parameters, in particular a fall in fibrinogen to below detectable levels, a gradual increase in PTT, and a dramatic terminal increase in prothrombin time, which are believed to be markers of endothelial cell activation.

The main histopathological features of AHXR are endothelial swelling or disruption, vascular thrombosis with blood extravasation, interstitial edema, and features of tissue injury (9,10). Deposition of immunoglobulins (IgM and IgG), complement and fibrin on the vascular endothelium is variable. In some cases, cellular infiltration (e.g., neutrophils, macrophages, T cells, and/or NK cells) can be seen, but this is again variable. Whether the cellular infiltration is entirely due to AHXR, but could also indicate a superimposed acute cellular response (as seen in allotransplantation) remains unclear.

The exact mechanisms underlying the development of AHXR remain uncertain, but the evidence suggests that antibody plays a significant role, although complement activation may be less important (or even of no importance). The presence of antibody activates the vascular endothelium, increasing expression of tissue factor and various adhesion molecules, and inducing a procoagulant state. Antibody-dependent cell-mediated cytotoxicity (ADCC) is thought to be a likely mechanism for injury to the cells, with the presence of antibody stimulating cellular infiltration. The approaches that have been successful in preventing HAR may therefore not be entirely successful in preventing AHXR. For example, although antibody depletion successfully prevents HAR

and may delay AHXR, it is impractical to perform extracorporeal immunoadsorption indefinitely. Other approaches to preventing or reversing AHXR are therefore being investigated.

Although natural (T cell-independent) anti-Gal antibody is present at a variable level before the transplant, the presence of a transplanted pig organ or cells leads to a T cell-dependent induced antibody response (11). This primarily takes the form of a 100-300-fold increase in anti-Gal IgG and the development of antibody to new non-Gal pig antigens. It is believed that this induced response plays a major role in the development of AHXR, yet there is evidence to suggest that AHXR develops even when the induced antibody response is successfully prevented (12).

4.3. **Acute Cellular Rejection**

In vitro evidence suggests that the cellular response to a pig xenograft will be at least as strong, if not stronger, than that towards an allograft (13). The cellular response *in vivo*, however, remains poorly defined, largely because AHXR has not yet been successfully overcome. Pino-Chavez has described the microscopic features of a cell-mediated response that develops even in the presence of intensive immunosuppressive therapy (10). However, conclusions regarding the pure cell-mediated response (in the absence of AHXR) must be interpreted with great caution as data are extremely limited. It would seem likely, however, that even if AHXR can be overcome, a cell-mediated response will develop (14), although some of the therapy used to prevent AHXR is also likely to suppress the acute cellular response.

4.4. Chronic xenograft rejection

Chronic rejection is poorly understood even in allotransplantation and, as no long-term survival has been achieved so far in pig-to-primate models, we know virtually nothing about it in XTx. However, it appears likely that chronic rejection, e.g. transplant vasculopathy, will develop in transplanted pig organs, possibly more rapidly than in allotransplants.

Because of the need for prolonged intensive immunosuppressive therapy to suppress the cell-mediated response and the probable early development of chronic rejection, the barriers to XTx may be overcome only by the induction of immunological tolerance. Our own center has been investigating the possibility of inducing tolerance to xenografts for several years.

5. TOLERANCE

Tolerance can be defined functionally as a specific absence of an immune response to an antigen. However, over the last several years it has become clear that tolerance is not confined to the absence of a response, but may involve an active and continuous down-regulation of several responses, and may be brought about by various mechanisms. Therefore, operationally, tolerance can be defined as the survival of an organ transplant in the absence of exogenous immunosuppression. The immunologically-tolerant transplant recipient maintains stable graft function, yet retains immune reactivity to all other antigens, and is not at risk from the complications associated with chronic nonspecific immunosuppressive therapy.

Several approaches to the induction of xenogeneic tolerance are being explored.

5.1. Mixed hematopoietic cell chimerism

On the basis of successful organ Tx between fully MHC mismatched allogeneic nonhuman primates, resulting in stable kidney allograft function for several years without immunosuppression (15), attempts to induce tolerance by mixed hematopoetic cell chimerism have been undertaken in the pig-to primate model.

The Tx of xenogeneic bone marrow or mobilized hematopoetic progenitor cells obtained by leukapheresis may provide an approach to the induction of donor-specific tolerance towards a xenogeneic organ. The strategy is to partially ablate the host's lymphohematopoetic system and to replace it with that of the donor through bone marrow (BM) Tx. The sucessful induction of mixed hematopoetic chimerism will, even if not maintained long-term, secure central robust tolerance to an organ of the BM donor.

Two types of macrochimerism resulting from BM Tx into a preconditioned host can be distinguished - (I) full chimerism, in which the entire lymphohematopoetic system of the recipient is destroyed by myeloablative therapy and replaced by donor cells, which leads to complete donor hematopoetic reconstitution (16), and (ii) mixed chimerism, which results in a state of coexistence of both recipient and donor lymphohematopoetic cells in the recipient (17, 18), and which can be achieved by milder (nonmyeloablative) forms of treatment which do not totally ablate the host's lymphohematopoetic system.

Several studies in allogeneic animal models have shown that donor-specific tolerance can be induced by this approach even across major histocompatibility (MHC) barriers (15,19). However, to date it has been possible to use this approach to induce clinical Tx tolerance only in selected patients, largely because of the nonhematopoetic toxicity which is associated with the conditioning regimen and, in particular, the significant risk of graft-versus-host disease (GVHD) following HLA-mismatched BM Tx (20).

In concordant rodent models, nonmyeloablative host conditioning has been demonstrated to induce mixed hematopoetic cell chimerism and tolerance (21,22). In comparison to allogeneic Tx, in XTx a more extensive preconditioning of the host by the administration of monoclonal antibodies against NK cells and T cells is necessary (23), as these cells appear to play a more significant role in resisting the engraftment of xenogeneic than allogeneic BM. Allogeneic BM may be partially protected from NK cell-mediated resistance because of NK cell surface inhibitory receptors that cross-react on multiple class I alleles (24). Seebach and Waneck have showed that inhibitory receptors may not interact well across species barriers (25), whereas some NK-activating receptors may function between species (26,27).

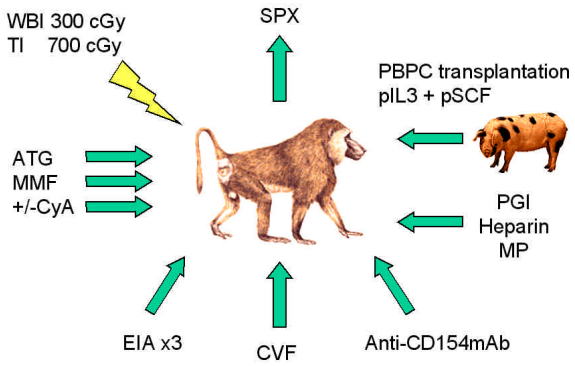

Figure 1. Basic non-myeloablative regimen for induction of mixed chimerism and tolerance in the pig-to-baboon model. SPX = splenectomy, WBI = whole body irradiation in two fractions (each of 150 cGy), TI = thymic irradiation, ATG = anti-thymocyte globulin, MMF = mycophenolate mofetil (by continuous i.v. infusion), CyA = cyclosporine (by continuous i.v. infusion), EIA = extracorporeal immunoadsorption, CVF = cobra venom factor, MP = methylprednisolone, PG1 = prostacycline, pIL3+pSCF = porcine hematopoietic growth factors. Transplantation of pig mobilized peripheral blood progenitor cells (PBPC) is carried out on days 0, 1 and 2.

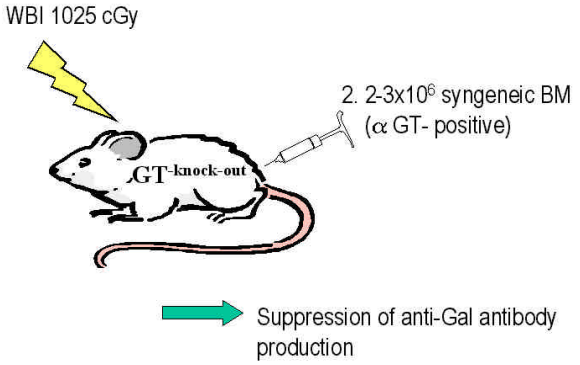

Figure 2. Molecular chimerism in mice. Reconstitution of αGT-knockout mice with syngeneic BM transduced with a retrovirus carrying a functional αGT gene suppresses production of anti-Gal antibodies. WBI = whole body irradiation, αGT = α-galactosyltransferase, BM = bone marrow.

BM Tx between discordant species is impeded by the presence of natural xenoreactive antibodies. In αGT knock-out mice, which lack a functional αGT, as do humans, and produce anti-Gal antibodies, both preexisting and newly-developing Gal-reactive B cells can be tolerized by the induction of mixed chimerism by tranplantation of Gal-expressing allogeneic or xenogeneic BM (28,29). Furthermore, induction of mixed chimerism is able to prevent HAR, AHXR, and cell-mediated rejection of primarly vascularized cardiac xenografts (29).

Attempts have been undertaken to induce hematopoietic chimerism in pig-to primate models. This has proved much more challenging in this species combination. Long-term chimerism has been only occasionally achieved at levels detectable by polmerase chain reaction (31). Innate immune barriers and species disparities with regard to adhesion molecules and cytokines may by critical barriers to xenogeneic BM Tx (32,33). So far, this barrier can only be partially overcome, even with the administration of donor species-specific cytokines (34) and the induction of costimulatory blockade by an anti-CD 154 mAb.(11).

Bühler and coworkers of our center have shown that blocking of the T-cell induced antibody response by an anti-CD154 mAb (Figure 1) may help in prolonging chimerism , but consistant chimerism is lost after the first month (11). After the i.v. infusion of large numbers of pig mobilized hematopoetic progenitor cells (obtained by leukapheresis), mixed chimerism was detected by flow cytometry in two baboons. In one animal, in which no anti-Gal IgG could be detected for 30 days, pig cells were detected by flow cytometry on days 16-22 (maximum 6 % on day 19), and pig colony forming cells were detected on days 19-33. Microchimerism was continuous by polymerase chain reaction for 33 days. These results suggest that there is no absolute barrier to pig hematopoetic cell engraftment in primates, although reproducible methods of producing this have not yet been developed.

5.2. Molecular Chimerism

Although perhaps the most reliable method of inducing allogeneic tolerance is the achievement of mixed hematopoietic cell chimerism, there are several factors that currently make this approach difficult for inducing tolerance to the Gal epitope or to pig non-Gal antigens in primates. These relate primarily to the difficulty of establishing porcine BM engraftment and long-term lymphohematopoiesis in primates (35). Building on the powerful concept of mixed chimerism, another approach has been developed, so-called “molecular chimerism”. This addresses the possibility of employing gene therapy to modify the host’s immune response to the Gal antigen, and is based on the genetic modification of autologous BM.

Bracy *et al.* have shown that the induction of molecular chimerism (following reconstitution of αGT-knockout mice with syngeneic (wild-type) BM transduced with a retrovirus carrying a functional αGT gene) suppresses production of anti-Gal antibodies (Figure 2). Subsequently, they have demonstrated that efficient transduction and expression of a retrovirally-transduced αGT gene in host BM-derived cells induces stable long-term tolerance to the Gal epitope (36). These mice remained tolerant to Gal even when challenged by extensive immunization with pig cells. Analysis of B cells from reconstituted mice using immunogobulin ELISPOT assays indicated that Gal-reactive B cells are eliminated from the immunological repertoire.

Although this approach has been successful in αGT-knockout mice, it has to date not been successful in the pig-to-baboon model, mainly because of the difficulty of adequately transducing sufficient numbers of baboon BM cells (Teranishi *et al.* unpublished data).

5.3. Transplantation of Thymic Tissue

An alternative approach is the transplantation of xenogeneic thymic tissue. This obviates the requirement for engraftment of donor hematopoetic progenitor cells and does not depend on a functioning host thymus for achieving T cell recovery. Sykes *et al.* have shown the capacity of xenogeneic thymic tissue to reconstitute functional host T cells in thymus-deficient or thymectomized mice (37-39). Fetal pig thymus and liver fragments (as a source of hematopoetic cells) were implanted under the kidney capsule of normal C57BL/10 (B10) mice that had been thymectomized and depleted of T and NK cells (37). Mature mouse $CD4^+$ T cells developed in the pig thymus grafts and repopulated the peripheral lymphoid tissues. The fetal pig thymic grafts grew markedly. No anti-pig IgG response was produced. Mixed lymphocyte reactions confirmed that the new T cells were functional and were tolerant to pig antigens. These mice showed long-term acceptance of donor-pig skin grafts while rejecting both allogeneic and xenogeneic third-party skin grafts. Both porcine and murine histocompatibility class II cells were detected in long-term thymus grafts, and T-cell repertoire analyses suggested that tolerance to both donors and recipients had developed, at least in part, by intragraft clonal deletion. This study provided the first demonstration that donor-specific skin graft tolerance can be induced across widely-disparate (discordant) species barriers.
Although transplantation of non-vascularized fetal porcine thymic grafts into baboons receiving a regimen of T cell depletion and thymectomy seems to be able to induce temporary specific hyporesponsiveness to pig antigens, the thymic grafts showed no evidence of long-term engraftment (40).

Based on the experience that vascularized thymic allotransplants are able to induce rapid and stable tolerance to class I-disparate pig kidney allografts in thymectomized recipients (41), attempts have been undertaken to apply this strategy to the induction of xenogeneic tolerance in the pig-to-baboon model. However, Yamada *et al.* from our center have demonstrated that transplanting a vascularized, composite thymokidney (a kidney with vascurarized thymic tissue within the capsule) into a baboon permits the survival of donor thymic epithelium. In one experiment, although the thymokidney was rejected by T cell-independent antibodies, necessitating excision of the graft, mixed lymphocyte reaction showed donor-specific hyporesponsivness for more than 2 months after withdrawal of immunosuppression (Bath RN *et al.*, manuscript in preparation).

This approach, although clearly successful in allograft models, has been hampered largely by the presence of T cell-independent xenoreactive antibodies which lead to rejection of the graft. If the native anti-Gal antibody production could be suppressed, it is likely that thymokidney or vascularized thymic Tx would lead to T cell tolerance and an absence of an induced antibody response.

5.4. Costimulatory Blockade

The costimulatory pathway of CD40 and its T cell ligand CD154 is crucial for effective activation of T cells to antigen (42) and plays an important role in establishing T cell-dependent B cell activity (43). The use of costimulatory blocking reagents has attracted considerable interest for the induction of an anergic state of tolerance. Long-term survival of vascularized grafts and islet grafts in rodents can be obtained by blocking the CD40 pathway alone or in combination with blockade of the B7/CD28 pathway. Although T cell costimulatory blockade with anti-CD154 mAb and CTLA4Ig has profound immunosuppressive effects in various Tx models (44-50), costimulatory blockade alone has not induced tolerance in the stringent test of primary skin grafts across full MHC barriers in euthymic recipients. Only thymectomized mice accept skin grafts permanently after treatment with anti-CD154 mAb and donor splenocytes (50).

In a primate kidney alloTx model, treatment with CTLA4Ig in combination with an anti-CD154mAb was highly effective in preventing acute cellular rejection and in prolonging graft survival. However, *in vitro* reactivity persisted against donor antigens, and the evidence is that indefinite graft survival would not be achieved without chronic treatment with costimulatory blockade agents. However, based on BM Tx in combination with costimulatory blockade, a protocol has been developed that is able to achieve mixed chimerism in fully MHC-mismatched BM Tx without irradiation, cytotoxic drugs or depleting antibodies (51)

At our center, we have investigated anti-CD154 mAb extensively in pig-to-primate models of hematopoetic progenitor cell and organ Tx. It has not been possible to achieve tolerance in these models. However, when administered with mycophenolate mofetil, anti-CD154 mAb has been able to block the induced antibody response to both Gal and non-Gal porcine epitopes. This observation confirms that the induced antibody response in XTx is T cell-dependent, whereas the baseline natural antibody production, which continued despite anti-CD154mAb therapy, is T-cell-independent. Furthermore, as mentioned above, anti-CD154mAb treatment substantially prolonged mircrochimerism in baboons receiving miniature swine hematopoetic cell transplants.

6. CONCLUSIONS

Xenogeneic hematopoetic cell, gene, and vascularized thymus Tx represent promising approaches to induce humoral and/or cellular tolerance across species barriers, which might make XTx a clinical reality. The major challenges in this field include the development of specific and nontoxic methods for overcoming the immunological and physiological barriers to engraftment of xenogeneic hematopoetic progenitor cells and, in particular, a means of suppressing natural xenoreactive antibody production while tolerance is induced.

The goal of tolerance might be achieved if genetic engineering of the donor pig, allowed for the generation of αGT-knockout pigs. The absence of targets for anti-Gal antibodies should make porcine tissues more

capable of surviving and facilitate engraftment of pig hematopoietic cells.

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Abbreviations: AHXR = acute humoral xenograft rejection, BM = bone marrow, FACS= flow cytometry, HAR = hyperacute rejection, mAb = monoclonal antibody, MHC = major histocompatibility complex, Tx = transplantation, XTx = xenotransplantation

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