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Normal Development in Porcine Thymus Grafts and Specific Tolerance of Human T Cells to Porcine Donor MHC

Boris Nikolic,* Jason P. Gardner,2 David T. Scadden,† J. Scott Arn,* David H. Sachs,* and Megan Sykes3*‡

The induction of T cell tolerance is likely to play an essential role in successful xenotransplantation in humans. In this study, we show that porcine thymus grafts in immunodeficient mice support normal development of polyclonal, functional human T cells. These T cells were specifically tolerant to MHC Ags of the porcine thymus donor and responded to nondonor porcine xenontigens and alloantigens. Exogenous IL-2 did not abolish tolerance, suggesting central clonal deletion rather than anergy as the likely tolerance mechanism. Our study suggests that the thymic transplantation approach to achieving tolerance with restoration of immunocompetence may be applicable to xenotransplantation of pig tissues to humans. The Journal of Immunology, 1999, 162: 3402–3407.

Materials and Methods

Transplantation procedures

Fetal human tissue was provided by the Anatomic Gift Foundation (Laurel, MD) in accordance with a Massachusetts General Hospital Institutional Review Board-approved protocol. C.B.-17 scid/scid (H-2b) mice were purchased from the Department of Radiation Oncology at Massachusetts General Hospital (Boston, MA). Fetal porcine tissue was harvested from pregnant sows, which were obtained from our MHC-defined miniature swine colony maintained at Tufts University School of Veterinary Medicine (Grafton, MA) (5). SLA4 (mAb was used as thymic donors. PBL were harvested from the MHC-matched SLA and the fully MHC-mismatched SLA pigs. Human fetal thymic and liver fragments (gestational week 17) and second trimester (gestational day 50–72) miniature swine fetal thymic fragments (0.5 x 0.5 x 1 mm) were transplanted under the kidney capsule via a midline laparotomy incision. All surgical procedures, including harvest of porcine and human tissue, were performed under sterile conditions as described (3). The SCID mice were maintained in microisolator cages, and 2 h before transplantation, they received 3-Gy whole body irradiation.

Immunohistochemistry

Sections of 4-μm thickness were prepared from frozen THY/LIV grafted tissue, and staining was performed as described (3). Primary Abs used for staining were as follows: 34-2-12 (mouse IgG2 anti-H2D); 25-9-17 (mouse IgG2 anti-1-A9); 2.27.3a (mouse IgG2 anti-SLA class I public determinant); and 1053H2-18-1 (mouse IgG2 anti-pig class II). Isotype-specific negative control mAb was HOPC-1 (mouse IgG2a). Biotinylated secondary mAb was rat anti-mouse IgG2a (PharMingen, San Diego, CA). After washing, the specimens were incubated with Avidin DH and biotinylated horseradish peroxidase (reagents A and B, respectively; Vectorstain ABC kit; Vector Laboratories, Burlingame, CA), followed by 3-amin-9 ethylcarbazole substrate (Aldrich, Milwaukee, WI), and were counterstained with hematoxylin.

Monoclonal Abs and flow cytometry (FCM)

Human and porcine PBL were prepared by centrifugation over a Ficoll layer. Human lymphocytes were isolated from the spleens as described (3). The following mAbs were used for staining: FITC-RPA-T4 (anti-human CD4), FITC-RPA-T8 (anti-human CD8), biotin-RPA-T4 (anti-human CD4), biotin-RPA-T8 (anti-human CD8), biotin-HIT3a (anti-human CD3), FITC-B159 (anti-human CD56) (all anti-human mAbs were purchased from PharMingen), FITC-MSA-4 (anti-porcine CD2), biotin-MSA-4, FITC-74-2-12 (anti-porcine CD4), biotin-74-2-12, FITC-76-2-11 (anti-porcine CD8), biotin-89HII-6-15 (anti-porcine CD3) (all anti-porcine mAbs were purchased in our laboratory), biotin-34-2-12 (anti-mouse H-2D), FITC-HOPC-1, and biotin-HOPC-1 (mouse IgG2a, as negative controls) (PharMingen). After 30 min of incubation with FITC mAbs, cells were washed and stained with biotinylated mAbs, followed by two washes and a 10-min phycocerythrin-streptavidin incubation. The anti-human, anti-mouse, and anti-porcine mAbs were used at dilutions previously shown to

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be optimal for staining, without significant interspecies cross-reactivity. Nonviable cells were excluded using the vital nucleic stain propidium iodide.

**RT-PCR analysis**

mRNA was prepared from 5 × 10⁶ cells using guanidium thiocyanate and oligo(dt) spun columns (QuickPrep mRNA purification kit, Pharmacia, Piscataway, NJ) before DNaseI (Promega, Madison, WI) digestion. Iso- rated mRNA was quantified spectrophotometrically and 300 ng was incubated with random hexanucleotide primers and Moloney reverse tran- scribed (Life Technologies, Grand Island, NY) to prepare cDNA in 80-μl reactions. Amplification was performed using 2.5 μl of cDNA product in each PCR (25 μl) with 1.25 U Taq DNA polymerase (Pharmacia) and 0.4 μM of each oligonucleotide sequence. TCR Vβ subset analysis was performed as previously described using a panel of primers specific for individ- ual human Vβ-chains (6). All samples were positive for the low molecular size PCR product of GSα, a constitutively expressed gene with intervening intron that allows discrimination of contaminating genomic DNA (7). Samples with contaminating genomic DNA were excluded from subsequent analysis. Specificity was confirmed by Southern blot hybrid- ization with digoxigenin-labeled internal probe at 65°C for 4 h using 5× SSC, 0.5% SDS, and 1% blocking solution (Boehringer Mannheim, Indiana- polis, IN). Blots were washed sequentially under high stringency with 3× SSC and 0.5% SDS at room temperature, 1× SSC and 1% SDS at 37°C, and 0.1× SSC and 1.0% SDS at 65°C, followed by incubation with an antidigoxigenin Ab alkaline phosphatase conjugate and detection by chemiluminescence. Parallel extractions were performed from samples without RT or with water alone to exclude DNA contamination.

**Lectin stimulation**

Thymic grafts were carefully disaggregated, passed through a mesh sieve, and washed twice in PBS. FACs staining confirmed that >95% thymo- cytes were of human origin (data not shown). Thymic cells were resus- pended in complete medium consisting of Iscove’s modified Dulbeco’s medium (Mediatech, Washington, DC) containing 20% FCS (Sigma, St. Louis, MO), glutamine (1 mM), penicillin (10 IU/ml), and streptomycin (10 mg/ml), and aliquots of thymocytes were dispensed into 96-well tissue culture plates at a density of 2.5 × 10⁵ cells per well, stimulated with either 2 μg/ml Con A (Sigma) or 5 μg/ml phytohemagglutinin (Murex, Dartford, U.K.) in the presence of human rIL-2 (20 IU/ml; Chiron, Emeryville, CA), and incubated at 37°C in 5% CO₂. Cultures were pulsed with 1 μCi (1 Ci = 37 Gbq) of [³H]thymidine (DuPont/NEN, Boston, MA) on the fourth day, harvested on the fifth day with a Tomtec (Orange, CA) automated har- vestor, and assayed in a Pharmacia LKB Betaplate.

**MLR**

Human thymocyte suspensions were prepared from human/human (HU/ HU) and swine/human (SW/HU) grafts and washed in AIM-V medium (Life Technologies, Gaithersburg, MD) supplemented with 10% (v/v) hu- man serum and 1% HEPES. Triplicate wells containing 4 × 10⁵ responders with 4 × 10⁶ allogeneic human PBL stimulators (30 Gy irradiated), or 4 × 10⁵ xenogeneic SLA cc or SLA dd PBL stimulators (30 Gy irradiated) in a total volume of 0.2 ml of medium were incubated at 37°C for 4 days in 5% CO₂. Human rIL-2 (250 IU/ml) (Aldesleukin; Chiron) was added where indicated. Cultures were pulsed with 1 μCi of [³H] on the third day, har- vested on the fourth day with a Tomtec automated harvester, and assayed in a Pharmacia LKB liquid scintillation counter.

**Results**

**Porcine thymus supports normal maturation of human thymocytes**

Since the thymus transplantation approach might have the poten- tial to induce xenotolerance in humans, we assessed the ability of fetal porcine thymus to support human thymopoiesis when co- transplanted with fetal human liver (as a source of hematopoietic progenitors) under the kidney capsule of SCID mice (SW/HU SCID). Control animals received human fetal thymus with human fetal liver (HU/HU SCID) or porcine fetal thymus with porcine fetal liver (SW/SW SCID). Additional control groups received hu- man fetal thymus (HU THY SCID), human fetal liver (HU LIV SCID) or porcine fetal liver (SW LIV SCID) alone. By 13 wk postimplantation, thymus/liver grafts had undergone considerable growth over the initial size of 0.5 × 0.5 × 1 mm of implanted tissue fragments. Examples of HU/HU (10 × 8 × 5 mm, containing 30 × 10⁶ human thymocytes) (left), SW/SW (10 × 5 × 5 mm, containing 30 × 10⁶ porcine thymocytes) (middle) and SW/HU grafts (25 × 15 × 10 mm, containing 290 × 10⁶ human thymocytes) (right) harvested at 13 wk in a single experiment are shown in Fig. 1A. HU fetal liver (FL) or SW FL grafts, when implanted without thymic tissue, did not show evidence of growth. HU THY tissue grafted alone appeared small and fibrous at 13 wk, which is consistent with previous reports showing that a source of lymphoid
progenitors must be provided to maintain such grafts (8). Histological analysis revealed a lobulated structure, as well as rich vasculature in HU/HU, SW/SW, and SW/HU grafts. SW/HU grafts showed identical microscopic anatomy to that of HU/HU grafts and normal human thymus. The origin of cells populating the SW/HU grafts was determined by immunohistochemical staining and by FCM. As shown in Fig. 1B (upper panel), SW/HU grafts contained porcine class I\(^{1}\) and class II\(^{1}\) cells in a net-like structure. The majority of thymocytes were human (\(\geq 90\%\) of total cells), Fig. 2). It was previously shown that murine H-2\(d\)-expressing cells could induce tolerance of developing T cells in HU/HU SCID mice (8–10). As shown in Fig. 1B (lower panel), porcine and murine class I\(^{1}\) and class II\(^{1}\) cells with a dendritic morphology were detected, mainly in the corticomedullary junction and the medulla of SW/HU grafts. Consistent with the possibility that these murine cells could induce tolerance of human T cells to host Ags, no evidence of graft-vs-host disease, autoimmune disease, or wasting syndrome was detected in any SW/HU SCID mice.

Thymocytes from SW/HU grafts showed an indistinguishable FCM profile from those of HU/HU grafts, and from those of normal human thymus, with characteristic proportions of double positive (DP), single positive (SP) CD4, SP CD8, and double negative thymocytes (Fig. 2A). The CD4:CD8 ratio of human SP cells ranged between 2 and 3.5:1. The acquisition of CD3 was typical of normal thymopoiesis (Fig. 2A). In some animals, a small population of porcine DP and SP CD4\(^{+}\) and CD8\(^{+}\) lymphocytes (Fig. 2A, bottom right panel) and a subpopulation of porcine CD3\(^{-}\) class I\(^{-}\) cells (data not shown) coexisted with human thymocytes.

**Human T cells that develop in porcine thymus are polyclonal**

Thymic epithelial cells are known to influence the shape of the TCR repertoire of developing thymocytes by mediating positive and, in some circumstances, negative selection. To assess the diversity of the TCR repertoire of human thymocytes developing in porcine and human thymus, we evaluated the expression of 25 human TCR V\(\beta\) chain transcripts by RT-PCR, using a panel of PCR primers for individual V\(\beta\) subsets (6). In the example shown in Fig. 2B, human thymocytes developing in human thymus expressed 24 of 25 V\(\beta\), which is consistent with studies showing comparable TCR V\(\beta\) diversity in HU/HU SCID mice to that in normal human thymus (11). Importantly, human thymocytes developing in porcine thymus grafts expressed a polyclonal TCR repertoire, with 21 of 25 V\(\beta\) being represented (Fig. 2B). This broad V\(\beta\) representation was observed in two independent experiments, and no particular V\(\beta\) subset was consistently undetectable.

Porcine thymocytes developing in porcine thymus did not express transcripts that cross-reacted with human V\(\beta\) primers (Fig. 2B), which excludes the possibility of porcine cells contributing to the observed human TCR polyclonality.

**Efficient human peripheral T cell reconstitution from porcine thymus grafts**

By 10 wk posttransplantation, human SP T cells were found to circulate in the peripheral blood of SW/HU and HU/HU recipients (Fig. 3). Furthermore, in 12 of 13 SW/HU recipients, a small number of porcine SP T cells coexisted with human SP T cells in the circulating pool (Fig. 3, A and B). The recipients of THY grafts...
alone had the lowest levels of T cells in PBL, which reached undetectable levels by 15 wk postimplantation. HU or SW FL grafts alone, implanted without THY tissue, did not support T cell development (Fig. 3A). The circulating human T cells showed a normal CD4:CD8 ratio (2–4:1) (Fig. 3B), and the majority of CD4+ cells (85%) had the naive CD45RA high phenotype (data not shown). The largest number of human SP CD4+ and CD8+ lymphocytes was detected in PBL of SW/HU SCID mice (human PBL in SW/HU vs HU/HU, p < 0.001), as well as in grafts (human T cells in SW/HU vs HU/HU, p < 0.005), spleens, and peritoneal cavities of this group (Fig. 3C). In five different experiments involving 30 SW/HU and 20 HU/HU recipients at several different time points, we observed that swine thymus supports higher levels of human T cell maturation than human thymus.

**Human T cells that develop in porcine thymus are functional and specifically tolerant of porcine donor MHC Ags**

To determine the functional status of T cells developing in SW/HU SCID thymic grafts, human T cells were stimulated with Con A and phytohemagglutinin. Human lymphocytes in porcine thymus grafts or mouse spleens showed substantial proliferative responses to lectin stimulation (stimulation indices >50). Con A-stimulated thymocytes from HU/HU and SW/HU grafts showed similar up-regulation of the early activation markers CD25 and CD69 at 24 h (data not shown). To further assess the function of human T cells that developed in thymic xenografts, we performed allogeneic anti-human MLR. Fig. 4 shows that human lymphocytes that developed in HU/HU grafts mounted similar alloresponses to those of human lymphocytes that developed in the SW/HU grafts.

The marked growth of porcine thymic grafts detected in SW/HU SCID mice suggested that human T cells developing in these grafts might be tolerant to porcine Ags. To demonstrate tolerance of human T cells, we performed xenogeneic anti-porcine MLR. Human thymocytes from three of three SW/HU SCID grafts showed a complete lack of responsiveness to stimulators that were MHC-matched to the porcine thymus donors (SLAdd). However, these responder populations demonstrated normal proliferative responses against fully SLA-mismatched (i.e., nondonor) xenogeneic SLA stimulators (Fig. 4). Thus, tolerance was specific for the SLA of the donor pig. Also, human lymphocytes from the spleens of SW/HU animals were tolerant (data not shown). In contrast, lymphocytes from HU/HU SCID grafts mounted similar responses to both SLAcc and SLAaa xenogeneic stimulators (Fig. 4). The addition of exogenous human rIL-2 (Fig. 4) did not induce anti-SLAaa responses by human T cells that developed in SW/HU grafts, suggesting central clonal deletion rather than anergy as a
mechanism of tolerance. This deletion is likely to be a consequence of the presence of pig class II$^{\text{high}}$ cells of dendritic morphology, which were observed in the long-term SW/HU thymic grafts (Fig. 1B). IL-2 did not increase the response against third party SLA$^{\text{cc}}$ by human T cells tolerized toward SLA$^{\text{dd}}$ (Fig. 4), perhaps due to tolerance of cells responding to xenodeterminants shared by SLA$^{\text{cc}}$ and SLA$^{\text{dd}}$ pigs. We have observed a similar phenomenon in xenogeneic rat—mouse bone marrow transplantation, in which induction of murine T cell tolerance toward the donor rat strain, although specific, is associated with partial hypersensitivity to third party rat (12).

Discussion

We report here a new way to achieve xenogeneic pig-specific tolerance among human T cells. We show that porcine fetal thymic grafts can support the development of functional, normal human T cells, from hematopoietic precursors provided by human fetal liver cells. These human T cells show specific immunological tolerance to porcine xenoantigens, while showing full responsiveness to allogeneic human stimulators and MHC-mismatched (i.e., nondonor) porcine Ags. Our data suggest that tolerance occurs through a clonal deletion mechanism in the porcine thymus. These results establish the principle that human T cell tolerance can be induced to a discordant xenogeneic porcine donor.

The development of a tolerant human T cell repertoire in our porcine thymic grafts occurred in the presence of class II$^{\text{high}}$ cells of porcine origin that had a dendritic morphology. Some or all of these are likely to be dendritic cells, which have potent ability to induce deletional tolerance in the thymus (13, 14). It was reported from the HU-SCID mouse model that tolerizing human MHC class II$^{\text{high}}$ dendritic-like cells have a finite life span (10), which might lead to a late failure of deletional tolerance, if a similar disappearance of porcine dendritic cells occurred. However, we have observed that porcine “dendritic-like” cells persist long-term (>30 wk) in porcine thymic tissue grafted to B10 or BALB/c nude mice, even if the pig thymus is grafted without pig fetal liver or any other porcine stem cell source (4). Thus, porcine thymic tissue may contain long-term progenitors of these cells, or the “dendritic-like” cells may themselves be long-lived, and studies are in progress to distinguish between these possibilities. In addition, even if porcine dendritic cells were to disappear with time from swine fetal thymus/human fetal liver grafts, this might not lead to a failure to tolerize subsequently developing T cells, since porcine thymic epithelial cells may also serve this function, albeit by a different mechanism (9, 15). If lasting tolerance were still not achieved at a sufficient level, it is possible that the application of this approach could require repeated injections of hematopoietic cells from the porcine donor.

The ability of the thymus to regenerate T cells after clinical treatment with myeloablative or T cell-depletion protocols in the transplant setting is limited in adult human recipients, in whom thymic function progressively declines with increasing age (16–18). The use of thymic transplantation to generate a new functional T cell repertoire could overcome this potential limitation in older human recipients and provide tolerance for donor tissues. Several studies of allogeneic thymic transplantation in congenitally athymic humans have been successful (19, 20). Our study indicates that the thymic transplantation approach to achieving tolerance with restoration of immunocompetence could be applied to xenotransplantation to humans from donors such as pigs, from which potentially unlimited amounts of donor thymus tissue could be obtained.

Since MHC restriction is generally believed to be determined by the MHC of the thymus, it might be predicted that immunoincompetence would occur due to mismatches between the class II MHC restriction imposed by donor allogeneic or xenogeneic thymus and the MHC of host-type APC present in the periphery (21). However, thymectomized mice in which mouse T cells develop in porcine thymic grafts have shown excellent host-restricted immune responses, including the ability to clear Pneumocystis carinii infections (22). Similarly, results following allogeneic thymic transplantation for human congenital thymic aplasia (DiGeorge syndrome), in which the MHC of the donor thymus and of host hematopoietic cells differ markedly, suggest that this “restriction incompatibility” may not be a major obstacle to the achievement of adequate immune function (19).

The superior ability of porcine thymus to reconstitute human T cells in SCID mice may be due to several factors, including species differences in T cell “set points”, or to the fact that porcine thymic tissue was implanted on the day it was harvested, whereas human thymic tissue was received one day following its removal from the fetus. In view of more recent results (not shown), in which superior reconstitution of human T cells was not observed in SW mice compared with human thymus, we believe that tissue quality may be the most important factor determining efficiency in this regard.

The SW/HU transplant recipients provide, to our knowledge, the only existing model in which porcine and human cells coexist long term in vivo. Thus, it provides an excellent in vivo model in which to address the possibility of transmission to and possible pathogenic effects of porcine retroviruses and other infectious agents on human cells, a possibility that has recently generated considerable concern (23, 24). Studies of this kind are now in progress using tissues from the SW/HU transplanted mice.

In addition to inducing tolerance, the xenogeneic thymus-grafting approach might also contribute to the treatment of patients infected with HIV. One of the target organs of HIV-1 infection is the thymus, which undergoes severe damage with epithelial injury and marked impairment of thymopoiesis (25–28). Thymic injury due to HIV infection, combined with the normal postpubertal thymic involution existing in many HIV-infected patients, could result in a failure to generate new CD4$^{+}$ T cells to replace those that are destroyed in the periphery, even with adequate retroviral suppression (29–31). Porcine thymi appear to be resistant to HIV infection (M. Sykes and S. Stanley, unpublished observations). Therefore, with effective antiretroviral therapy administered at the time of porcine thymic implantation, adequate human thymopoiesis might be achieved in porcine thymic xenografts. Thus, xenogeneic thymic transplantation has the potential to provide an important adjunct to the therapy of HIV-infected patients.

If this approach is to be applied for tolerance induction in a clinical setting, there will be a need for a host-conditioning regimen that would deplete donor-reactive T cells. Several nonmyeloablative-conditioning approaches for tolerance induction have recently been described, including one that allows engraftment of porcine thymic tissue in immunocompetent mice (4, 32). Thus, we are optimistic that clinically feasible and safe approaches to applying these findings are likely to be developed. Furthermore, it is possible that in advanced HIV patients, who have extremely low numbers of clonally expanded T cells, these conditioning requirements would be even lower. This possibility is currently being evaluated in SIV-infected monkeys.

In summary, this study shows that the thymic transplantation approach to achieving tolerance with immunocompetence could be applied to xenotransplantation from porcine donors to humans.
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References