PREVENTION OF THROMBOTIC MICROANGIOPATHY IN PIG CARDIAC GRAFTS IN BABOONS

SPECIFIC AIM

The proposed studies are directed towards resolving a major problem in pig-to-nonhuman primate (NHP) heart transplantation (Tx), namely the development of thrombotic microangiopathy (TM) in the graft (associated with thrombocytopenia and a reduction in fibrinogen in the recipient) and/or consumptive coagulopathy (CC). The exact causative factors of TM and CC remain uncertain, but (i) innate immunity (antibody, complement, platelets, macrophages, NK cells), (ii) the adaptive immune response (T and B cells), and (iii) molecular incompatibilities between pig and NHP may all play roles.

We shall investigate coagulation dysfunction in baboons receiving an immunosuppressive regimen that prevents the adaptive immune response which, in the presence of a graft from a pig genetically engineered to be protected from the innate response, allows coagulation dysfunction to be investigated without other complicating factors.

The source of the hearts will be α1,3-galactosyltransferase gene-knockout (GTKO) pigs expressing two human complement-regulatory proteins (CD46 and CD55) and two human coagulation-regulatory proteins (thrombomodulin [TBM] and endothelial cell protein C receptor [EPCR]). GTKO CD46 CD55 TBM EPCR pigs have not been available to any group previously.

In Aim 1, we shall carry out heterotopic heart transplantation (n=5) and, as graft and recipient survival is anticipated to be for several months, in Aim 2 we shall carry out orthotopic heart Tx (n=5) to determine that the grafts are able to support the circulation. The proposed studies should provide data that will advance progress towards the clinical introduction of pig heart Tx. In Aim 1, if the graft fails or TM develops, we shall investigate the potential mechanisms contributing to its development, and take steps to address these. Aim 2 will be aimed at confirming that graft and recipient survival is sufficient to warrant a carefully-planned clinical trial.

BACKGROUND AND SIGNIFICANCE

Xenotransplantation has immense potential to solve the critical need for organs, tissues, and cells for clinical Tx (Ekser 2012). The increasing availability of genetically-engineered pigs is enabling progress to be made in pig-to-NHP models. Heterotopic hearts from transgenic pigs have survived for 6-8 months in NHPs (Kuwaki 2005; Tseng 2005; Mohiuddin 2012). However, despite the prevention of rejection, coagulation dysfunction between pig and NHP has proven to be the major problem for graft and recipient survival, and can result in TM (Figure 1), resulting in myocardial fibrosis and graft failure, and/or life-threatening CC with systemic bleeding manifestations (Buhler 2000b; Houser 2004; Kuwaki 2004, 2005; Tseng 2005; Shimizu 2008; Ezzelarab 2009; Lin CC 2010).

Coagulation dysregulation

Thrombin generation is a constant feature of the immune response to xenografts in NHPs, evidenced both clinically as CC and histopathologically as TM (Houser 2004; Ezzelarab 2009). In addition to being a strong pro-coagulant factor and a potent platelet activator, thrombin and its receptors are being recognized not only as pro-inflammatory, but also as stimulators of the innate response (Niessen 2008; Ezzelarab 2012a). At our own center, Ezzelarab et al (2012a) have demonstrated that thrombin activates the T cell response to pig cells, and it is believed this is an important factor contributing to an increased xenoreactive response (Figure 2). Furthermore, the activated vascular endothelial cells (ECs) (Lin CC 2008) and the generated thrombin subsequently activate platelets, leukocytes, and other inflammatory cells in the recipient, initiating a vicious cycle. We believe T cell activation by thrombin will be significantly
reduced by the expression of human TBM (and reduced further by additional expression of EPCR) on the pig graft.

Physiologic incompatibilities between the coagulation systems of pig and primate are problematic (Robson 2000; Lawson 1997; Schulte am Esch 2001; Dorling 2001; Cowan 2008; Chen D 2005; Lin CC 2009) and probably explain the mechanistic basis of coagulation pathway dysregulation in organ xenoTx. Determination of the exact mechanism by which TM and CC are initiated is important because it may enable further genetic modification of the pig or suggest therapy that might prevent them.

Increasing data suggest that the immune response may influence the development of coagulation disorders and, equally, procoagulant factors and platelet activation may in a reciprocal manner amplify the immune response.

Of particular importance to this study is the observation that porcine TBM inefficiently amplifies the thromboregulatory protein C pathway, a prothrombotic effect that could be corrected in vitro by expression of human TBM (and preferably by additional expression of EPCR) (Figure 3).

The introduction of these genes may overcome the coagulation incompatibilities between pig and primate, and forms the basis of these proposed studies. The current proposal will therefore address this important barrier to successful heart Tx in NHPs. It will provide essential data, particularly relating to platelet aggregation and thrombin generation, on the effect of the expression of TBM and EPCR on the development of TM and CC.

PRELIMINARY STUDIES

We shall review 4 major areas of recent interest.

1. **Generation of genetically-engineered pigs:**
   A number of genetically-engineered pigs are now available to us for testing in our pig-to-NHP models (through our collaborators at Revivicor, Blacksburg, VA). GTKO.CD46.CD55.TBM.EPCR pigs will be available early in 2013.

2. **Immunosuppressive regimen:**
   For years, our standard regimen in the pig-to-NHP model included anti-CD154mAb, but because of its thrombogenicity, we have developed an alternative regimen that is equally effective in preventing an adaptive immune response/sensitization (Table 1) (Ezzelarab 2012b; Iwase et al, unpublished).
3. **In vitro platelet aggregation assay:**

Using a Chrono-log Whole Blood Aggregometer (Iwase 2012), we have measured platelet aggregation after incubation of blood with human aortic EC (hAEC) and pAEC from wild-type (unmodified, WT), GTKO, and various transgenic pigs expressing complement- or coagulation-regulatory proteins. In addition, we have previously tested numerous anti-platelet agents *in vitro* and *in vivo* in baboons (Alwayn 2000; Appel 2001a,b). Platelet aggregation induced by GTKO pAEC or by pAEC expressing human complement-regulatory proteins (CD46 and/or CD55) and/or human coagulation-regulatory proteins (tissue factor pathway inhibitor or TBM or EPCR) was significantly reduced, particularly by GTKO.CD46.TBM pAEC (WT 53% aggregation vs GTKO.CD46.TBM 28%; p<0.01) though not to the minimal level of aggregation induced by hAEC (5%) (Iwase H, manuscript submitted) (Figure 4). The data suggested that the Tx of a heart from a GTKO.CD46.TBM pig would be associated with no (or delayed) development of TM in the graft. (It is of interest that EPCR had some effect, as it primarily enhances the effect of TBM (Figure 3). In this assay, it enhanced the effect of pig TBM, as the pig cells did not express human TBM. Its enhancement of human TBM should be much greater.)

<table>
<thead>
<tr>
<th>Table 1: Immunosuppressive regimen currently used in pig-to-NHP studies</th>
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<td>Agent / Dose</td>
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<tr>
<td>1. Anti-thymocyte globulin (ATG) 1-10mg/kg x2 (days -3 and -1) to reduce the CD3(^+)T cell count to &lt;500/mm(^2) by day of Tx</td>
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<td>2. Anti-human CD40mAb 25mg/kg i.v. on days -1, 0, 4, 7, 10, 14, and weekly.</td>
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<td>3. Belatacept 20mg/kg i.v. on days -1, 0, 4, 7, 14, and every 2 weeks.</td>
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<td>3. Rapamycin i.m. to maintain a level of 8-12ng/ml.</td>
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<td>4. Methylprednisolone 5mg/kg/day i.v. or i.m. on the days of ATG administration and on day 0 (day of Tx), tapering to 0.5mg/kg/day i.m. by day 5, and to 0.25mg/kg/day i.m. by day 30, after which 0.25mg/kg i.m. is administered on alternate days until day 60, when it is discontinued.</td>
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4. **Coagulation dysfunction in pig-to-NHP heart Tx**

When hearts from pigs not expressing a coagulation-regulatory protein are transplanted into baboons, features of TM (fall in platelet count and fibrinogen level) occur within the first 3 weeks (Figure 5), and at the time of graft failure or euthanasia of the baboon, histopathological features of TM are present. We have recently carried out 3 heart transplant from GTKO.CD46.TBM pigs (not expressing CD55 or EPCR) (Iwase et al. unpublished). In contrast to all previous pig-to-baboon heart transplants, there was no thrombocytopenia or reduction in fibrinogen (Figure 5). Two experiments are ongoing at >3 months, but one baboon was euthanized on post-Tx day 52, when there were no histopathological features of TM.
Figure 4: Effect of genetic modifications of pAEC on in vitro human platelet aggregation

![Figure 4](image)

Figure 5: Platelet counts and fibrinogen levels in baboons following Tx of hearts from GTKO.CD46.CD55 pigs (mean of 4) or from a GTKO.CD46.TBM pig (mean of 3).

![Figure 5](image)

RESEARCH DESIGN AND METHODS

General Methods

Animal Model

The pig-to-baboon heterotopic heart Tx model has been a model of choice for pre-clinical studies of xenoTx for several years (Cooper 1988; Kuwaki 2005; Tseng 2005; Ezzelarab 2009; Ekser 2012). As pigs have many features that make them suitable as sources of organs for human recipients if the immunological barriers can be overcome, the experience gained in the pig-to-baboon model will prove valuable for subsequent clinical xenoTx trials.

Procedures

Vascular access: Pigs have blood drawn only under anesthesia at the time of organ excision. Baboons have two venous catheters inserted, and are fitted with a jacket and tether which allows access for blood withdrawal and drug infusion (Cooper 1994). Because of the risk of bleeding and infection, these catheters will be removed 2 weeks post-Tx, after which the baboons will be sedated x1-2 weekly for blood draw and i.v drug administration.

Heterotopic (HHT) and orthotopic (OHT) heart transplantation: HHT is performed in the abdomen (Cooper 1994). OHT is performed using cardiopulmonary bypass, using the technique...
used clinically (Cooper 1983). All operative procedures will be carried out by Dr J. Bhama, an experienced clinical and experimental cardiac transplant surgeon.

Myocardial biopsy: The endomyocardium can be biopsied (after HHT or OHT) with a biopsy probe introduced through the femoral vein. Alternatively, after HHT, an open myocardial biopsy can be carried out through a small incision over the heart. Endomyocardial biopsies will be performed by Dr J. Teuteberg, who has experience of these techniques both clinically and experimentally.

Immunosuppressive and supportive therapy: The immunosuppressive regimen will be that currently used in our studies (Table 1). All baboons will receive prostacyclin (20ng/kg/min) and dopamine (2-7μg/kg/min) as ‘anti-endothelial-inflammatory’ agents for 48h. Ganciclovir (5mg/kg/day) as prophylaxis against cytomegalovirus infection will be administered for 3 months, and cimetidine (10mg/kg x2 daily) will be administered while i.v. catheters are in situ to prevent peptic ulceration. All baboons will also receive low molecular weight heparin (LMWH, 700IU/day s.c.) for 90 days and aspirin 40mg p.o. on alternate days indefinitely.

Study Endpoint and Euthanasia

Follow-up will be for 6 months. When HHT has been carried out, if heart graft failure develops (cessation of palpable contractions, from whatever cause), the heart will be excised under general anesthesia, and the baboon will be followed for at least 1 month for further study of antibody levels, recovery of T cell numbers, etc. In our experience, timely excision of the graft allows rapid recovery from coagulation dysregulation (Kuwaki 2005; Tseng 2005). The immune status of the baboon will be monitored for a further month while immunosuppressive therapy is maintained. A significant increase in anti-pig antibody level, particularly of IgG, after excision of the graft would indicate sensitization to pig antigens.

Laboratory Methods

Complete blood count and chemistry will be monitored initially x2 weekly and then less frequently.

Coagulation parameters that will be monitored include platelet count, platelet aggregation assay, platelet activation factors (PAF, BTG, sP-selectin), fibrinogen, INR, D-dimers, TAT complex, F1+2, FXa/TF activity, soluble TBM, antithrombin activity, PA1-tPA, thrombin generation, and histopathology (IHC/IF) for coagulation-related markers (see below).

Immune parameters that will be monitored include:-

Flow cytometry data on T and B cell numbers (CD3+, CD4+, CD8+, CD20+) will be acquired using a FACScan fluorescence cytometer (Becton Dickinson) and analyzed using Winlist mode analysis software (Verity Software House, Topsham, ME) (Ezzelarab 2012a). Baboon IgM and IgG binding to GTKO PBMC and pAEC will also be monitored by flow cytometry (Ezzelarab 2006b). The median fluorescence intensity (MFI) will be determined using FITC-conjugated goat anti-human IgM and IgG polyclonal Abs. Measurement of baboon complement activity will be by the CH50 test, and of baboon complement fractions, C3a/C5a, by ELISA (Ezzelarab 2009). In the mixed leukocyte reaction (MLR), baboon PBMCs and CD4+ T cells will be used as responders at a concentration of 0.2x10^6 cells/well, as previously described (Ezzelarab 2012b). Irradiated pAECs (with or without activation) will be used as stimulators at stimulator-responder ratios of 1:10, and 1:20; incubation will be for 5 days. ³H-thymidine (1μCi/well) will be added to each well during the last 16h of incubation. They will be analyzed by beta-scintillation counting on a liquid scintillation counter (PerkinElmer, Waltham, MA). The mean of at least triplicate results will be expressed as ³H-thymidine incorporation values.

Myocardial biopsies will be prepared for histopathological examination by light microscopy (hematoxylin and eosin, periodic acid-Schiff) and by direct immunofluorescence (Shimizu 2008; Houser 2004; Ezzelarab 2009, 2012a; Lin CC 2010). For conventional histology, tissues will be fixed in 10% formalin, embedded in paraffin. Five-micron thick sections will be
stained for immunohistochemical staining. Immunofluorescence will be performed on frozen sections, and electronmicroscopy on glutaraldehyde-fixed tissues. The following pathological markers will be studied: (i) Graft humoral and cellular rejection, e.g. IgM, IgG, C3, C4d, C5b-9, CD3, CD4, CD8, CD20, CD31, CD55, and CD68; (ii) Coagulation dysregulation and TM, e.g. platelets, fibrin, TF, vWF, CD39; (iii) Inflammation and endothelial cell activation, e.g. CD31, E-selectin, P-selectin, ICAM1, and VCAM1.

Aim 1: (Year 1) To investigate the coagulation disorders in baboons after HHT from GTKO.CD46.CD55.TBM.EPCR pigs (n=5).

Rationale:
With control of both the innate and adaptive immune responses (by specific gene manipulation of the organ-source pig and a proven immunosuppressive regimen), we hypothesize that the expression of both TBM and EPCR on pig vascular endothelium will prevent TM (Figure 3).

Experimental:

In Vitro: Using an established platelet aggregation assay, we will measure platelet aggregation induced by GTKO.CD46.CD55 pAEC expressing TBM+EPCR, which has not been possible previously. We anticipate the expression of TBM+EPCR will result in a further reduction of platelet aggregation (<28%) (see Figure 4).

In Vivo: Baboons will undergo HHT from GTKO.CD46.CD55.TBM.EPCR pigs. The immunosuppressive regimen will be that detailed in Table 1. All baboons will be followed by frequent clinical and laboratory monitoring of immune and coagulation parameters (see General Methods). Graft function will be monitored by twice weekly palpation of the abdomen to determine the strength of contractions of the graft (0 to 3+). Echocardiography will be carried out monthly. Myocardial biopsies will be taken at 3 months, with euthanasia and necropsy at 6 months, and will indicate any features of rejection or of TM and/or graft vasculopathy (although these latter two features may not always be seen on biopsies) (Houser 2004; Kuwaki 2005; Ezzelarab 2009).

If graft failure occurs through the development of TM, or if CC develops and cannot be reversed, the heterotopic heart will be excised, and the baboon will be followed for at least 4 weeks (see General Methods).

Anticipated Results and Alternative Plans:
We cautiously anticipate that all of the 5 hearts will continue beating in the recipients for >6 months.

Antibody-dependent complement-mediated EC injury will be prevented by the use of hearts from GTKO pigs expressing two complement-regulatory proteins (Ezzelarab 2009; Mohiuddin 2012, McGregor 2012; Iwase, unpublished data). Early features of antibody-mediated complement injury will be absent or rare.

With the transgenic expression of both TBM and EPCR, at the very least we anticipate a significant and prolonged delay in the onset of TM. On the basis of our recent experience transplanting hearts from GTKO.CD46.TBM pigs (Figure 5), coagulation parameters are unlikely to indicate a hypercoagulable state. However, if there are any features of such a state, we will be able to identify what parameters are affected, and, importantly, which factors are graft-derived and which recipient-derived. Platelet studies by flow cytometry and aggregation assays might show features of early activation. We anticipate we shall not see gross clinical features of CC (spontaneous bleeding, etc) unless the graft is about to fail from advanced TM. We anticipate that, at necropsy, the heart will show no or very mild features of TM (Houser 2004; Kuwaki 2005; Ezzelarab 2009).
The data will enable us to determine whether further genetic manipulations of the pig, e.g., expression of CD39, tissue factor pathway inhibitor, or hemeoxygenase-1 (all of which will be available during 2013-14), and/or increased administration of anti-thrombotic agents is indicated.

In the unlikely event that the expression of both TBM and EPCR results in a lack of viability or poor health of the organ-source pigs or in a bleeding diathesis after the pig heart has been transplanted into the recipient baboon, or if for any other reason pigs expressing both TBM and EPCR are not available to us, we will transplant hearts from GTKO.CD46.CD55.TBM pigs, which we know do not result in health problems for either pig or baboon. Transplanted hearts from these pigs have shown protection against TBM, though we anticipate an even greater protection if EPCR is also expressed (Figure 3).

If 4 out of 5 hearts do not function for 6 months and/or do not show histopathological features of TM, we shall not proceed to Aim 2 but will expand the studies under Aim 1 by transplanting hearts from GTKO.CD46.CD55.TBM.EPCR pigs that also express TFPI and/or CD39 and/or HO-1.

Aim 2: (Year 2) To investigate function and survival of hearts from GTKO.CD46.CD55.TBM.EPCR pigs after OHT in baboons (n=5).

Rationale:

Coagulation dysregulation is currently the sole remaining major barrier to successful pig heart Tx in NHPs. We anticipate that this barrier will be overcome in Aim 1 by the Tx of hearts from pigs expressing two coagulation-regulatory proteins (TBM and EPCR). Before considering a clinical trial of pig heart Tx (possibly initially as a bridge to alloTx), it will be essential to confirm that hearts from these pigs will support the circulation of baboons following OHT.

Experimental:

Baboons will undergo OHT from GTKO.CD46.CD55.TBM.EPCR pigs. The immunosuppressive regimen will be that detailed in Table 1. All baboons will be followed by frequent clinical and laboratory monitoring of immune and coagulation parameters (see General Methods). Graft function will be monitored by twice weekly palpation of the femoral pulse and auscultation, and by echocardiography on post-Tx days 2, 7, 14, 28, and then monthly or when there are any clinical features of cardiac dysfunction. Endomyocardial biopsies will be taken at 4 and/or 8 months, with euthanasia and necropsy at 12 months, and will indicate any features of rejection or of TM and/or graft vasculopathy (although these latter two features may not always be seen on biopsies) (Houser 2004; Kuwaki 2005; Ezzelarab 2009). A chest radiograph will be taken at 1, 4, and 8 months.

Anticipated Results and Alternative Plans:

The surgical personnel at our center have extensive experience of OHT in humans and NHPs (Cooper 1983), and we do not anticipate technical complications. There have been reports of initial poor function of pig hearts after OHT in baboons (McGregor 2009, and personal communication), but this is thought to be associated with an immediate immune injury when there has been inadequate protection from antibody and/or complement deposition. We anticipate that this dysfunction will be avoided or minimized by transplanting hearts from GTKO pigs expressing two complement-regulatory and two coagulation-regulatory proteins.

Nevertheless, inotropic support is likely to be required during the first 48 hours. Furthermore, we have clear evidence from studies in humans (Novitzky 1988, 1989; Cooper 2009) and baboons (Cooper 1983, 2009; Iwase 2012 unpublished) that there is a depletion of circulating free triiodothyronine (T3) after surgery involving cardiopulmonary bypass, which is associated with impaired myocardial function. We have reported extensive data indicating that
the administration of T3 prevents or reverses this myocardial dysfunction (reviewed by Cooper 2009). We believe these measures will protect against early graft dysfunction.

There is some preliminary evidence that there is a reduced incidence of TM after OHT than after HHT (McGregor 2009, and personal communication). The results of Aims 1 and 2 will determine whether this observation is correct.

If TM is prevented, the most likely cause of graft failure will be graft atherosclerosis, probably associated with the continuing presence of low levels of natural anti-pig (nonGal) antibodies. We will give consideration to methods of depleting such antibodies at intervals, e.g., by plasmapheresis, and/or by the administration of agents that deplete B or plasma cells, e.g., an anti-CD20mAb (Alwayn 2001; Mohiuddin 2012) and/or bortezomib (Everly 2009).

It is possible that, although after HHT a non-life-supporting pig heart graft will function well for periods >12 months, after OHT minor injury to the myocardium or the development of graft atherosclerosis will result in earlier graft failure. The study will therefore provide very important data with regard to whether a clinical trial of pig heart xenoTx should be contemplated. Nevertheless, the proposed studies will certainly provide data which will advance progress towards the clinical introduction of pig heart Tx.

ETHICAL CONSIDERATIONS:

All animal care procedures will be in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). Protocols have been approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh (IACUC protocol # 0902831A-1).

No non-animal model can indicate whether pig hearts could function in humans, but the use of baboons as surrogates for humans will provide such an indication. Cell lines and small animal models will not allow for the necessary methodology needed to test the genetically-engineered pig organs that will be transplanted in the proposed studies. In order for the Tx of genetically-modified pig hearts into humans to become a clinical reality, utilization of a nonhuman primate model is necessary. Only Old World primates, such as baboons, produce natural anti-pig antibodies similar to those produced by humans. Furthermore, the effect of costimulation blockade agents, as used in the proposed studies, has been investigated in nonhuman primates, but less comprehensively in other large animals.

The studies are justified by the continuing deaths of patients awaiting organ Tx which occur because the number of deceased human organs available for clinical Tx remains totally inadequate.