Published in final edited form as:

Xenotransplantation. 2012 September ; 19(5): 311–316. doi:10.1111/j.1399-3089.2012.00718.x.

HUMAN T CELL PROLIFERATION IN RESPONSE TO THROMBIN-ACTIVATED GTKO PIG ENDOTHELIAL CELLS

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Abstract

Background—Thrombin formation is a key feature in the activation of coagulation in pig xenograft recipients. As thrombin is known to activate endothelial and immune cells, we explored whether thrombin activation of pig endothelial cells (EC) was associated with an increased human T cell response.

Methods— α 1,3-galactosyltransferase gene-knockout (GTKO) pig aortic EC (pAEC) were activated by porcine interferon-gamma (pIFN γ), human (h)IFN- γ , or thrombin. Swine leukocyte antigen (SLA) class-I and -II expression were measured. Human PBMC and CD4⁺Tcell proliferation in response to activated pAEC, the effect of thrombin on pig CD80/CD86 mRNA, and the effect of thrombin inhibition by hirudin were evaluated.

Results—After pAEC activation, SLA-I expression did not change, and *only* pIFN γ upregulated SLA-II expression. PBMC proliferation to pIFN γ - and thrombin-activated pAEC were significantly higher ($p < 0.001$ and < 0.01) than to non-activated pAEC. CD4⁺T cell proliferation to pIFN γ - and thrombin-activated pAEC were significantly higher ($p < 0.001$ and < 0.01) than to non-activated pAEC. Thrombin inhibition by hirudin reduced thrombin-induced upregulation of pAEC CD86 mRNA, and significantly reduced human PBMC proliferation to pAEC in comparison to thrombin alone ($p < 0.05$).

Conclusions—Thrombin upregulates CD86 mRNA on pAEC, which is associated with increased human T cell proliferation against pAEC. Hirudin reduces CD86 mRNA in thrombin-activated pAEC, and is associated with downregulation of the human T cell proliferative response. The transplantation of organs from GTKO pigs transgenic for human thrombomodulin, and/or endothelial protein C receptor, in addition to therapeutic regulation of thrombin activation may reduce the cellular response to a pig xenograft and thus reduce the need for intensive immunosuppressive therapy.

Keywords

α 1, 3-galactosyltransferase gene-knockout; CD86; Endothelial cells; Pig; T cells, human; Thrombin; Xenotransplantation

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DISCLOSURE

David Ayares is an employee of Revivicor, Inc. The other authors declare they have no conflict of interest.

INTRODUCTION

Dysregulation of the coagulation system in organ xenograft recipients results in the development of a thrombotic microangiopathy in the xenograft and consumptive coagulopathy in the recipient (1, 2). Thrombin formation is a key feature in the activation of coagulation, which is upregulated following pig organ xenotransplantation (3).

Thrombin is a multifunctional serine protease, which is generated at sites of vascular injury. It is the most effective agonist for platelet activation, and considered the main effector protease of the coagulation cascade, resulting in clot formation. Recently, pro-inflammatory and immunostimulatory roles for thrombin have been recognized, i.e., roles unrelated to coagulation. Also, thrombin activates many cell types, including endothelial cells (4), and immune cells, notably monocytes, macrophages (5), and dendritic cells (6, 7).

Increased thrombin formation can result in activation of both the vascular endothelial cells of the pig xenograft and of the native endothelial cells of the recipient. We hypothesized that increased thrombin levels in organ xenograft recipients could induce activation of pig endothelial cells, with subsequent amplification of the primate recipient T cell response to pig antigens. If so, regulation of thrombin activation in xenograft recipients may be crucial for optimum control of adaptive and innate immune cellular responses. We evaluated *in vitro* the effect of stimulation of pig endothelial cells by high levels of thrombin on the human T cell response to pig antigens.

METHODS

Reagents

Thrombin (human-derived; Cat# T7009) and hirudin (cat# H7016) were purchased from Sigma-Aldrich (St. Louis, MO). Human IFN- γ and porcine IFN- γ were purchased from Serotec (Raleigh, NC). Purified mouse anti-swine leukocyte antigen (SLA) class I (Cat# 552547, clone 1E3), and class II DR (Cat# 553642, clone-1053h2-18-1) and FITC anti-mouse IgG_{2a/2b} (Cat# 553399, clone-R2-40) antibodies were purchased from BD Pharmingen (San Diego, CA).

Cells

Human peripheral blood mononuclear cells (hPBMC) were isolated from buffy coats of blood type O (Institute for Transfusion Medicine, Pittsburgh, PA). Human CD4⁺T cells were isolated using CD4⁺T cell isolation kit II (cat# 130-091-155, Miltenyi Biotec, Auburn, CA). Pig aortic endothelial cells (pAEC) were obtained from freshly-harvested α 1,3-galactosyltransferase gene-knockout (GTKO) porcine aortas; pAEC were then cultured and used from passages 3 to 8.

Mixed lymphocyte reaction (MLR)

Human PBMC and CD4⁺T cells were used as responders (0.2×10^6 cells/well). pAEC were stimulated with either thrombin (40U/mL), pIFN- γ (40U/mL), or human (h)IFN- γ (200U/mL) for 24h. Responder-stimulator pairs were not identical in any of experiments. Irradiated pAEC were used as stimulators at stimulator:responder ratios of 1:10 or 1:20. MLRs were harvested after 5 days. ³H-thymidine (1 μ Ci/well) was added to each well during the last 16h of incubation. Cells were harvested on glass-fiber filter mats with a cell harvester, and were analyzed by beta-scintillation counting on a liquid scintillation counter (PerkinElmer, Waltham, MA). Samples were tested in quadruplicate and the mean of ³H-thymidine uptake was calculated as counts per minute (cpm).

Flow cytometry

Using LSR II flow, pAEC were assessed for SLA class I and II expression before and after activation. Data were analyzed using WinMDI software (The Scripps Research Institute, San Diego, CA, USA), and relative geometric mean of fluorescence (GMF) was calculated by dividing GMF of each sample by GMF of the Isotype antibody.

Reverse transcription polymerase chain reaction (RT-PCR)

Pig CD80 and CD86 cDNA levels were measured using SuperScript III One-Step RT-PCR System from Invitrogen (Grand Island, NY, Cat # 12574-030). Primers used were pig-CD80 5'-TCTGTTTCAGGCATCGTTCAG-3' (forward) and 5'-CTCATACTTGGGCCACACCT-3' (reverse); pig CD86 5'-TTTGGCAGGACCAGGATAAC-3' (forward) and 5'-GCCCTTGTCTTGGATTGAA-3' (reverse); pig-GAPDH 5'-GGGCATGAACCATGAGAAGT-3' (forward) and 5'-TGTGGTCATGAGTCCTTCCA-3' (reverse). Thermal cycling conditions were 1 cycle of 55°C for 30 min, followed by 1 cycle [at 94°C for 2min], followed by 40 cycles at 94°C for 15min/55°C for 30min/68°C for 1min, followed by 68°C for 5min on Eppendorf Mastercycler.

Statistical analysis

Statistical analysis was carried out using the Student t-test. Values of $p < 0.05$ were considered significant.

RESULTS

Thrombin activation of GTKO pAEC is associated with increased hPBMC and CD4⁺T cell proliferation in MLR

In MLR, the proliferation of both human PBMC and CD4⁺T cells in response to non-activated and activated GTKO pAEC was measured (Figure 1). pAEC were activated using thrombin (40U/mL), pIFN γ (40U/mL), or hIFN γ (200U/mL). At a 1:10 stimulator:responder ratio, the hPBMC proliferation in response to pIFN γ -activated and thrombin-activated pAEC was significantly higher ($p < 0.001$ and $p < 0.01$, respectively), than that to non-activated pAEC. Human PBMC proliferation in response to hIFN γ -activated pAEC was only slightly increased. At a 1:20 stimulator:responder ratio, hPBMC proliferation to activated and non-activated pAEC showed a similar pattern.

Similarly, at a 1:10 stimulator:responder ratio, human CD4⁺T cell proliferation (Figure 1) in response to pIFN γ -activated and thrombin-activated pAEC was significantly higher ($p < 0.001$ and $p < 0.001$, respectively) than that to non-activated pAEC, while human CD4⁺T cell proliferation in response to hIFN γ -activated pAEC was only slightly increased.

Thrombin activation of GTKO pAEC does not upregulate SLA II expression

Upregulation of SLA I and II on pAEC results in increased human CD8⁺T and CD4⁺T cell proliferation, respectively (8). We evaluated the expression of both SLA I and II on GTKO pAEC before and after activation with thrombin, pIFN γ , or hIFN γ (Figure 2). Before activation, GTKO pAEC constitutively expressed SLA I and minimally expressed SLA II. After activation, SLA I was upregulated after pIFN γ activation, but only slightly upregulated when activated by thrombin. While pIFN γ activation upregulated SLA II expression, no upregulation was detected after thrombin or hIFN γ activation. This indicates that the increased human CD4⁺T cell proliferation that follows thrombin activation of pAEC is not associated with upregulation of SLA II expression.

Thrombin modulates GTKO pAEC CD80 and CD86 mRNA levels

It is known that porcine CD80 and CD86 can costimulate human T cell proliferation (9). Thrombin activation of GTKO pAEC could induce proliferation of human T cells through upregulation of costimulatory molecules. We evaluated the levels of CD80 and CD86 mRNA before and after pIFN γ or thrombin activation (Figure 3). pIFN γ activation of GTKO pAEC upregulated both CD80 and CD86 mRNA. Similarly, thrombin activation resulted in the upregulation of both CD80 and CD86 mRNA. However, on some occasions, upregulation of CD80 was not as consistent as CD86. This suggests that the increased human PBMC and CD4⁺T cell proliferation in response to thrombin-activated pAEC is associated with increased CD28 costimulation of human T cells by activated pAEC.

Thrombin inhibition with hirudin is associated with reduced human PBMC and T cell proliferation

To further investigate the role of thrombin activation of pAEC on the human cellular response, GTKO pAEC were activated with thrombin (40U/mL) in the presence or absence of hirudin (20U/mL). hPBMC and CD4⁺T cell proliferation was significantly reduced when GTKO pAEC were incubated with both thrombin and hirudin ($p < 0.05$) rather than with thrombin alone (Figure 4). Furthermore, upregulation of CD86 mRNA in thrombin-activated GTKO pAEC was reduced when pAEC were incubated with thrombin and hirudin. Collectively, these data indicate that reduction or prevention of thrombin activation of pAEC is associated with reduced hPBMC proliferation, possibly due to reduced costimulation.

DISCUSSION

The availability of GTKO pigs (10, 11) has allowed hyperacute rejection to be minimized, with subsequent prolongation of survival of pig organs after transplantation into nonhuman primates. However, even in the absence of a measurable adaptive immune response, activation of coagulation remains a major problem, and results in graft failure from thrombotic microangiopathy and/or the development of a consumptive coagulopathy in the xenograft recipient (1, 2, 12, 13).

Thrombin is a serine protease that is generated from prothrombin at the sites of vascular injury, and mediates the final step in the coagulation cascade, which is fibrin production and clot formation. Increased levels of thrombin have been recorded after pig organ xenotransplantation (3). Upregulation of both pig (donor) and primate (recipient) tissue factor (2, 13), would result in activation of the coagulation cascade in xenograft recipients, and increased thrombin formation. It is known that thrombin can activate several cell types, including endothelial cells, platelets, monocytes and fibroblasts. It is likely that high levels of thrombin would result in activation of endothelial cells in the pig xenograft and the recipient “native” endothelial cells, as well as circulating immune cells. This would result in initiation of a loop of activation, leading to increased thrombin formation.

Recently, it has also been shown that thrombin or the activation of thrombin receptors has pro-inflammatory and immunostimulatory effects on immune cells. Thrombin induces HLA-DR and CD86 expression on dendritic cells, and thrombin-treated dendritic cells induce allogeneic T cell proliferation (6). Stimulation of thrombin receptors augments production of the chemokine, CCL18, by mature dendritic cells (7) and induces IL-8/CXCL8 expression in THP-1 monocyte cell line-derived macrophages and primary human macrophages (5). Additionally, thrombin is known to activate NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and to upregulate NF- κ B-dependent genes in pig endothelial cells (4). NF- κ B plays a key role in regulating cellular responses to inflammation. Thrombin activation may, therefore, play a crucial role in modulating the immune response to

xenografts through the activation of both xenograft endothelial cells and recipient immune cells.

We therefore hypothesized that, following organ xenotransplantation, prolonged high levels of thrombin can result in activation of pig vascular endothelial cells, and this in turn might amplify the human T cell response to pig antigens.

In vitro, we assessed the effect of high levels of thrombin on GTKO pAEC, and the response of human PBMC and T cells to the thrombin-activated pAEC. We found that human T cell proliferation was increased in the presence of thrombin-activated GTKO pAEC, which was not due to upregulation of SLA I or II. It is possible that SLA expression can be upregulated during contact of pAEC with human T cells. This may have an additional stimulatory effect on the proliferation of human T cells in response to thrombin-activated pAEC. We also found that thrombin activation modulated porcine costimulatory molecules, particularly CD86. It is noteworthy that upregulation of CD80 mRNA was not consistent among different GTKO pAEC. As RT PCR is not quantitative, quantitative measurement of CD80 and CD86 mRNA levels with qRT PCR will be needed.

In the context of cellular rejection of a xenograft, $\text{INF}\gamma$ is produced by the recipient (primate) effector T cells. We confirm that, even at high concentration, human $\text{INF}\gamma$ does not activate pAEC as strongly as thrombin. Furthermore, inhibition of thrombin with hirudin was associated with reduced human T cell proliferation and reduced CD86 mRNA expression.

Although in the present study we activated GTKO pAEC using a high concentration of thrombin (40U/mL) for 24h only, we suggest that, *in vivo*, thrombin formation for prolonged periods of time (even at low levels) might have a similar effect on the xenograft. Additionally, we used hirudin at 20U/mL aiming to achieve 50% reduction in thrombin-induced activation. However, a full dose-dependent analysis will be needed to determine the optimum dose of Hirudin.

In summary, in addition to its key role in coagulation, thrombin may result in the amplification of the recipient (primate) T cell response to pig antigens through its effect on CD86 upregulation. CD28/B7 costimulatory pathway blockade using CTLA4-Ig can successfully prevent human anti-pig T cell responses *in vitro* (14), which suggests that blockade of this pathway may prevent or inhibit the induced T cell response resulting from thrombin activation of pig endothelial cells *in vivo*. The effect of CD28/B7 costimulatory pathway blockade by CTLA4-Ig on T cell proliferation in response to thrombin-activated cells has yet to be determined.

If so, in addition to clinically-applicable immunosuppressive therapy, therapeutic regulation of thrombin formation or the development of pigs transgenic for thromboregulatory genes, e.g., human thrombomodulin and/or endothelial protein C receptor, may be necessary to successfully prevent the adaptive immune response to the pig antigens.

Porcine thrombomodulin is a poor cofactor for the activation of human protein C (15). Expression of human thrombomodulin and/or endothelial protein C receptor results in activation of protein C, a potent anticoagulant protein, and thus might restore an anticoagulant environment. In addition, as a result of endothelial cell activation, thrombomodulin and other anticoagulant proteins are shed from the cell surface; restoration of these molecules should help overcome the procoagulant state associated with acute humoral xenograft rejection.

Acknowledgments

Mohamed Ezzelarab is supported in part by the Shelly Patrick Fellowship at the Thomas E. Starzl Transplantation Institute. Work on xenotransplantation in the Thomas E. Starzl Transplantation Institute of the University of Pittsburgh is supported in part by NIH grants #U19 AI090959-01, #U01 AI068642, and # R21 A1074844, and by Sponsored Research Agreements between the University of Pittsburgh and Revivicor, Inc., Blacksburg, VA.

ABBREVIATIONS

GTKO	α 1,3-galactosyltransferase gene-knockout
IFN-γ	interferon-gamma
pAEC	pig aortic endothelial cells
PBMC	peripheral blood mononuclear cells
SLA	swine leukocyte antigen(s)

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Figure 1. Increased human PBMC and CD4⁺T cell proliferation in response to thrombin-activated pAEC

GTKO pAEC were activated using thrombin (40U/mL), pIFN γ (40U/mL), or hIFN γ (200U/mL), for 24h. The human PBMC (top) and CD4⁺T cell (bottom) proliferative responses to pIFN γ - and thrombin-activated GTKO pAEC were significantly higher than to non-activated pAEC (Stimulator:responder ratios of 1:10 and 1:20). Cell proliferation is presented as counts per minute (cpm). “Spontaneous” indicates responders alone. Data are representative of three different experiments. (*p<0.01, **p<0.001 in comparison to non-activated pAEC).

FIGURE 2A

FIGURE 2B

Figure 2. Thrombin does not upregulate SLA II expression on GTKO pAEC GTKO pAEC were activated using thrombin (40U/mL), pIFN γ (40U/mL), or hIFN γ (200U/mL) (A). SLA I expression was upregulated after pIFN γ , and slightly upregulated after thrombin activation. SLA II expression was upregulated only after pIFN γ activation, but not after thrombin or hIFN γ activation. The geometric mean of fluorescence (GMF) is indicated in each histogram. Isotype controls are represented by grey histograms. (B) Mean of relative GMF results (n=4) obtained following pAEC activation (error bars represent SEM).

Figure 3. Thrombin modulates CD80 and CD86 mRNA levels in GTKO pAEC

CD80 and CD86 mRNA levels were measured by PCR in GTKO pAEC before and after pIFN γ or thrombin activation for 24 hours. While CD80 mRNA was upregulated more than CD86 mRNA after pIFN γ activation, both CD80 and CD86 mRNA were upregulated after thrombin activation. Data are representative of two different experiments.

FIGURE 4A **FIGURE 4B** **Figure 4. Inhibition of thrombin activation by hirudin reduces human PBMC and T cell proliferation**

GTKO pAEC were activated using thrombin (20 and 40U/mL) with or without hirudin (20U/mL) for 24 hours (Figure 4A). Inhibition of thrombin with hirudin significantly reduced hPBMC (left) and CD4⁺T cell (right) proliferation in response to thrombin-activated pAEC (stimulator:responder ratio of 1:10). Additionally, hirudin reduced the upregulation of CD86mRNA by thrombin (Figure 4B). (* $p < 0.05$ in comparison to thrombin-activated pAEC).