Original Paper

Pig BMSCs Transfected with Human TFPI Combat Species Incompatibility and Regulate the Human TF Pathway *in Vitro* and in a Rodent Model

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Key Words

Xenotransplantation • Tissue factor/Factor VIIa • Tissue factor pathway inhibitor • Animal model • Bone marrow mesenchymal stem cells

Abstract

Background: The activation of tissue factor (TF) is one of the major reasons for coagulation dysregulation after pig-to-primate xenotransplantation. Tissue factor pathway inhibitor (TFPI) is the most important inhibitor of TF. Studies have demonstrated species incompatibility between pig TFPI and human TF. **Methods:** A pig-to-macaque heterotopic auxiliary liver transplantation model was established to determine the origin of activated TF. Chimeric proteins of human and pig TFPI were constructed to assess the role of Kunitz domains in species incompatibility. Immortalised pig bone marrow mesenchymal stem cells transfected with human TFPI were tested for their ability to inhibit clotting *in vitro*. **Results:** TF from recipient was activated early after liver xenotransplantation. Pig TFPI Kunitz domain 2 bound human FXa, but Kunitz domain 1 did not effectively inhibit human TF/FVIIa. Immortalised pig bone marrow mesenchymal cells (BMSCs) transfected with human TFPI showed a prolonged recalcification time *in vitro* and in a rodent model. **Conclusion:** Recipient TF is relevant to dysregulated coagulation after xenotransplantation. Kunitz domain 1 plays the most important role in species incompatibility between pig TFPI and human TF, and clotting can be inhibited by human TFPI-transfected pig BMSCs. Our study shows a possible way to resolve the incompatibility of pig TFPI.

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Introduction

The shortage of human donor livers limits clinical liver transplantation [1]. Xenotransplantation using transgenic pig organs may meet the increasing need for liver transplantation in humans [2]. However, coagulation disorders after liver xenotransplantation, manifested as thrombotic microangiopathy and consumptive coagulopathy, are now regarded to be the major barrier to successful liver xenotransplantations from pigs to humans [3–5].

The activation of tissue factor (TF) plays an important role in coagulation disorders after liver xenotransplantation [6–9]. However, the origin of TF in the pig-to-primate liver xenotransplantation model is controversial. In the study by Nagayasu et al. [7], pig TF was positive at the time of recipient death. *In vitro* studies also indicated that aortic endothelial cells were TF positive when exposed in human serum [10]. However, in Ekser's study [8], pig TF was negative in donor livers at 2 hours after transplantation, when thrombotic microangiopathy and consumptive coagulopathy began, although recipient peripheral blood mononuclear cells (PBMCs) were already human TF positive. In Lin's study of kidney xenotransplantation from pig to primate [11], although TF mRNA was positive in recipient platelets, PBMCs, and the native organs (heart and liver), it was negative on the donor organ (kidney). Lin's *in vitro* study also revealed that activated TF on recipient platelets and PBMCs caused dysregulation of coagulation [6]. Thus, the activation of recipient TF may be the main cause of coagulation dysregulation in the early period after liver xenotransplantation.

Tissue factor pathway inhibitor (TFPI) is the most important endogenous inhibitor of blood coagulation initiated by TF [12–14]. The α isoform (TFPI α) has 3 Kunitz-type domains [11]. Kunitz domain 1 (K1) inhibits the Factor VIIa (FVIIa)/TF complex, and Kunitz domain 2 (K2) binds FXa. Kunitz domain 3 also plays a role in FXa binding and participates in TFPI localisation to the endothelium via a glycosylphosphatidylinositol (GPI) anchor. Another spliced form of TFPI, called TFPI β , contains only K1 and K2 and is directly attached to endothelial cells by a GPI linkage. Despite the presence of mRNA, TFPI β protein has not directly been observed by western blot analysis in non-transfected cells [13]. Therefore, only TFPI α is discussed in this study. Kopp et al. [15] showed that inhibition of human TF by pig TFPI α is inefficient, which may partly explain coagulation dysregulation after xenotransplantation. However, Lee et al. [16] showed that recombinant pig TFPI efficiently inhibits human TF *in vitro*. Thus, the species incompatibility between pig TFPI and human TF, and the role of the Kunitz domains, remains unclear.

Materials and Methods

Animals

Wu Zhishan miniature pigs (n=3, 18–20 kg, males) that were genetically engineered as α -1, 3-galactosyltransferase knockouts (GTKO) were obtained from the Institute of Animal Science of the Chinese Agricultural Sciences Academy (Beijing, China). Tibetan macaques (n=3, 23–26 kg, male) were obtained from the Sichuan Academy of Medical Sciences (Chengdu, China). The blood type of all of the pigs was O and that of all of the macaques was A, as determined with a human ABO blood type assay kit (Yingke, China).

All of the animals were handled according to the Ministry of Health guidelines for the care and use of laboratory animals (GB 14925-2001), and all of the procedures were approved by the Laboratory Animal Ethics Committee of the Fourth Military Medical School.

Heterotopic auxiliary liver transplantation from GTKO pigs to Tibetan macaques

A heterotopic auxiliary liver transplantation model from GTKO pigs to Tibetan macaques was established to study the cause of clotting disorders and the origin of TF. A liver graft (left lobe, weight 205–220 g) was harvested from each donor pig (Fig. 1A). The graft-to-recipient weight ratios were 8.9%, 8.3%, and 8.5%. The recipients were anaesthetised with ketamine (15 mg/kg) and xylazine (1 mg/kg) before intubation. Under isoflurane inhalation anaesthesia, a midline abdominal incision and splenectomy of the recipient was performed so that the donor graft could be placed in the splenic recess. The recipient's left

renal vein was interrupted, the distal end was connected to the graft's portal vein, and the proximal end was connected to the graft's hepatic vein. Before reperfusion, microvascular anastomosis was performed to join the graft's hepatic artery and the recipient's splenic artery to re-establish the liver graft circulation. The bile of the donor liver was drained through the abdomen to visually assess liver graft function (Fig. 1B). After surgery, the recipients were extubated and recovered from anaesthesia. Computed tomography angiography was used to observe blood supply of donor and recipient livers 24 hours after xenotransplantation (Fig. 1C).

The immunosuppressive regimen was based on Kim et al. [17]. In brief, induction therapy was performed with 3 doses of thymoglobulin 3 days before transplantation in all cases. Cobra venom factor was used 1 day before surgery to deplete complement factors (CH50) to 5% or less of baseline levels. Immunosuppression was maintained with decreasing doses of methylprednisolone (starting at 10 mg/kg) and tacrolimus (serum levels 10–25 ng/ml from day –1). Biopsies from both recipient and donor livers were obtained before, 2 hours after, and 48 hours after transplantation. The recipients were euthanised when the mean arterial pressure was consistently below 40 mmHg. Blood samples were analysed for routine blood tests, chemistry, tacrolimus serum levels, and coagulation. All of the tests were performed once a day (and on other occasions as needed) in the clinical laboratory of Xijing Hospital.

Cell culture

Aortic endothelial cells (AECs) were harvested from GTKO pigs and cultured in 2% gelatin-coated tissue culture flasks with RPMI 1640 (HyClone, USA) containing 10% foetal bovine serum (FBS, HyClone, Logan, UT, USA), penicillin (0.5%), streptomycin (0.5%), and L-glutamine (2 mM) at 37 °C in 5% CO₂ [16]. Pig AECs were passaged six or fewer times for all of the experiments.

Pig bone marrow mesenchymal stem cells (BMSCs) were separated from the bone marrow of the spina iliaca of GTKO pigs and purified by density gradient centrifugation. Purified pig BMSCs were incubated in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12, HyClone) containing 15% FBS, penicillin (0.5%), and streptomycin (0.5%) at 37 °C in 5% CO₂.

In vitro model to induce the expression of human and pig TF

Adherent pig AECs and monocytes were pre-incubated with 5% human plasma for 8 hours to induce the expression of pig or human TF, respectively, on the cell surface [5, 18, 19]. Human plasma was drawn from volunteers with type A blood. After co-incubation, monocytes were isolated by centrifugation and AECs were harvested using 0.5% trypsin (Gibco, UK). For some tests, pig AECs or human monocytes were co-incubated with pig BMSCs, anti-TF (catalogue no. 17375, Abcam, USA), or TFPI for further studies.

Construction, expression, and purification of chimeric proteins

The pig TFPI sequence (NM_001135258.1) and human TFPI sequence (NM_006287.4) were obtained from GenBank. The TFPI sequence from Wu Zhishan miniature GTKO pigs was reported in Fang et al. [20]. TFPI sequences were compared using ClustaX2 software. The sequencing of all of the chimeric proteins, pig TFPI, and human TFPI was performed with molecular cloning, and the fragments were inserted into the pIRES2-EGFP expression vector (BD Biosciences Clontech, USA). A His tag was added to the C-terminus of chimeric and recombinant protein sequences for purification.

Chimeric proteins, recombinant human TFPI, and recombinant pig TFPI were expressed in *E. coli* BL21 cells. Isopropyl β-D-thiogalactoside (0.2 mM) was used to induce expression at 22 °C for 8 hours. Fusion proteins were purified with a Ni²⁺-nitrilotriacetate agarose resin (Qiagen, Germany), characterised by SDS-PAGE, and verified by western blotting.

TFPI activity assay

An actichrome TFPI activity assay kit (American Diagnostica, USA) was used to determine the activity of recombinant pig TFPI, recombinant human TFPI, and chimeric TFPI by measuring FX activation by the FVIIa/TF complex. Human FVIIa/TF (20 μl) was mixed with each source of TFPI (2.5 nM) and incubated at 37 °C for 30 minutes. Human FX (20 μl, Merck Millipore, USA) was then added to the mixture and incubated another 15 minutes at 37 °C. EDTA (20 μl) was added to terminate the reaction. Chromogenic detection was carried out using a highly specific chromogenic FXa substrate (American Diagnostica, Greenwich, CT, USA), and absorbance was measured at 405 nm. TFPI activity was determined by comparison with a standard curve generated using known concentrations of recombinant human TFPI in a 20-μl volume.

For assays of total cellular TFPI, cells were harvested with trypsin and lysed with 30 mM CHAPS (Amresco, Solon, OH, USA). Total protein was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Harvested cells were homogenised with a Brinkman Polytron homogeniser on ice and subjected to three freeze/thaw cycles in the presence of 1 mM 1-(L-transepoxy succinylleucylamino)-4-guanidinobutane (E-64, Sigma, St Louis, MO, USA). Cellular lysates were treated with 1 U/ml phosphatidylinositol-specific phospholipase C (PIPLC) for 1 hour at 37 °C. TF was removed by centrifugation at 436,000 ×g for 3 hours at 4 °C. TFPI activity was then determined as described by Mast et al. [14]. In some tests, the cells were pre-incubated with anti-human TFPI. All of the experiments were repeated three times.

FXa binding assay

The capacity of TFPI binding and the generation of thrombin initiated directly by FXa were determined as described [21]. Briefly, phospholipids (400 μM, 75% phosphatidylcholine, 25% phosphatidylserine vesicles, Sigma), co-factors (20 nM FV), and zymogens (5.6 μM prothrombin, 360 nM FIX, 680 nM FX) were incubated in 20 mM HEPES (pH 7.4) containing 150 mM NaCl and 2 mM CaCl₂. Prothrombin and coagulation factors were purchased from Merck Millipore (Germany). Immediately after mixing the phospholipids, co-factors, and zymogens with 2.5 nM of the desired source of TFPI (or no TFPI), FXa was added to initiate thrombin generation. The chromogenic substrate Spectrozyme TH (0.2 mM, American Diagnostica) was added to detect thrombin activity, and absorbance was measured at 405 nm. The concentration of prothrombinase was calculated from the rate of thrombin generation using a k_{cat} of 5016/minute. The inhibition of prothrombinase by TFPI was calculated by comparison with the control group carried out in the absence of TFPI. All of the experiments were repeated three times.

Recalcification assay

Human and macaque PBMCs were prepared by Ficoll-Hypaque density gradient centrifugation. After blood was diluted with the same volume of PBS, 10 ml of blood preparation was overlaid on 15 ml Ficoll-Hypaque (AppliChem GmbH, Germany). Centrifugation at 700 ×g was then performed to separate the buffy coat. The separated buffy coat interface was then added to 10 ml PBS and centrifuged at 300 ×g. After, the cells were suspended in α-MEM (HyClone, USA) containing 0.1% BSA. Human monocytes were selected from PBMCs by anti-human CD14 magnetic beads (Milenyi Biotec, Auburn, CA, USA) and macaque monocytes were selected by anti-nonhuman primate CD14 magnetic beads (Milenyi Biotec, Auburn, CA, USA). In brief, the cells were incubated with microbeads at 4 °C for 15 minutes, washed with MACS buffer, suspended and at last loaded on the top of a separation column. CD14+ cells were then selected using cold MACS buffer, centrifuged and resuspended in α-MEM (HyClone, USA) containing 0.1% BSA [8].

Pig AECs (1×10^5), human monocytes or recipient monocytes (1×10^5), or pig BMSCs (1×10^5) were suspended in 50 μl Tris-buffered saline. Normal human plasma (100 μl, Sigma-Aldrich) was mixed with one cell type or a combination of cells in a glass tube (Corning, NY, USA). CaCl₂ (250 nM in 10 μl Tris-buffered saline) and phospholipids (90 μl) were added. The tubes were agitated by continuous tilting, and the time required to form a fibrin clot was recorded. In some tests, the cells were pre-incubated with anti-human TF (1 mg/ml) or different sources of TFPI (2.5 nM) at 4 °C for 30 minutes before the recalcification assay.

Flow cytometry analysis

To identify BMSC surface antigens, 1×10^5 harvested cells were incubated in PBS containing 0.1% ChromPure pig IgG (catalogue no. 136756, Abcam, USA) and 1% NaN₃ on ice for 30 minutes. The following primary monoclonal antibodies were incubated with the cells for 30 minutes at 4 °C: anti-pig CD29 (catalogue no. 21845, Abcam, USA), anti-pig CD34 (catalogue no. 61737, GeneTex, USA), anti-pig CD45 (catalogue no. 28085, Abcam, USA), anti-pig CD105 (catalogue no. 53318, Abcam, USA), anti-pig CD80 (catalogue no. 11-0801-82, Sino biological, USA), anti-pig CD90 (catalogue no. 124527, Abcam, USA) and sheep anti-human TF (Affinity Biologicals, Canada). The cells were then washed with cold FACS buffer, suspended in 350 μl FACS buffer, and analysed on a dual-laser FACS Calibur (BD Biosciences, USA).

Atomic force microscopy (AFM)

Intermolecular forces were measured by AFM on a PicoSPM 5500 (Agilent Technologies, USA). AFM tips (MSCT, Si₃N₄, Veeco, USA) were functionalised with RCA₁₂₀. The thermal-noise mode was used to determine the spring constants of cantilevers. All of the experiments were performed in PBS with 1 mM

CaCl₂ and 0.5 mM MgCl₂. We collected more than 2000 force-distance cycles from different locations with each functionalised AFM tip. Matlab Version 7 (Math works, USA) was used to analyse the force-distance cycles.

Immunohistochemistry

The slides of BMSCs and histological liver sections were fixed with 4% paraformaldehyde and then incubated with the following primary antibodies at 4 °C overnight: mouse anti-human TFPI (catalogue no. 180619, Abcam), anti-human TF (catalogue no. 17375, Abcam), and anti-pig TF (raised against a synthetic peptide with the sequence IMRNVKETYV, which is present in pig TF). After washing, the sections were incubated with the appropriate secondary antibodies for 1 hour at room temperature. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI) and washed three times with PBS. Finally, the sections were mounted with Permount mounting medium (National Diagnostics, USA). Images were viewed with a Leica DM 750 light microscope (Leica, Germany).

Cell proliferation assay

A methyl thiazolyl tetrazolium (MTT) assay was used to determine cell proliferation. Primary and SV40T-transfected BMSCs were seeded at 3×10^4 cells/ml in triplicate wells of 96-well plates. Every 24 hours, 10 µl MTT (5 mg/ml, Sigma) was added to each well, and the cells were incubated for an additional 4 hours at room temperature. The supernatant was removed, and 150 µl DMSO was added to each well to solubilise the formazan crystals. The absorbance at 570 nm was measured on a microtitre plate spectrophotometer (BioTeck, USA).

Osteogenic and adipogenic differentiation of BMSCs

Pig BMSCs were seeded at 5×10^4 cells/well in 24-well plates containing osteogenic differentiation medium (Invitrogen, USA) or adipogenic differentiation medium (Invitrogen) for 28 days. The medium was changed every 48 hours. Following adipogenic differentiation, the cells were fixed with 4% paraformaldehyde and washed with double-distilled water. Lipid droplets were stained with 0.5% oil red-O in isopropanol for 20 minutes. The cells were washed with 60% isopropanol and double-distilled water and then observed under a light microscope. Following osteogenic differentiation, the cells were fixed at 4 °C in cold methanol and washed with DPBS without Ca²⁺ and Mg²⁺. The cells were stained with 5% silver nitrate and exposed to ultraviolet light for 1 hour. The cells were washed with double-distilled water and incubated with 5% sodium thiosulphate for 2 minutes. The staining of calcium nodules was observed with a light microscope.

Quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from each sample, and 3 µg were used for first-stand cDNA synthesis with oligo (dT) and Superscript III (Invitrogen). SYBR Green PCR Master Mix (Applied Biosystems) was used for PCR mixtures. The following primers were used [16, 22]:

SV40 T sense: 5'-CAAAGTGTGGTATGGCTGAT-3'

SV40 T antisense: 5'-GGCTACTGGGAAGTGA-3'

Human TFPI sense: 5'-GAATTCAAATAAGGCTGCGTA-3'

Human TFPI antisense: 5'-GAGGGAGCCTCAGAGTCGGCTTC-3'

Primate β-actin sense: 5'-CGGGAAATCGTCCGTGAC-3'

Primate β-actin antisense: 5'-TGGAAGGTGGACAGCGAGG-3'

Pig β-actin sense: 5'-CTCGATCATGAAGTGCAGT-3'

Pig β-actin antisense: 5'-GTGATCTCCTTCTGCATCTGTC-3'

Pig TF sense: 5'-TTTACCAACTCGCCCCCTTC-3'

Pig TF antisense: 5'-AATGTGCCGTTCACCCTGACTAAG-3'

Primate TF sense: 5'-GGAACCCAAACCCGTCATC-3'

Primate TF antisense: 5'-TCCGAGGTTGTCTCCAGGT-3'

Chimeric proteins were amplified with the same primers as pig TFPI. AmpliTaq Gold DNA polymerase was activated for 10 minutes at 95 °C, and amplification was performed for 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute on a Multicolour Real-time PCR detection system IQ 5 (Bio-Rad, USA). All of the tests were repeated three times.

Table 1. Graft type, immunosuppressive therapy, anticoagulants, anti-inflammatory agent, outcome and cause of death/euthanasia in pig to Tibetan macaques liver xenotransplantation

#	Type of pig	Immunosuppressive therapy	anticoagulants	anti-inflammatory agent	Survival (hours)	Cause of death / euthanasia
ZHQ01	GTKO	TAC, MMF, ATG, Anti-CD154 mAb, CVF	salviae miltiorrhizae	methylprednisolone	52	Pulmonary edema caused by fluid overload
ZHQ03	GTKO	TAC, MMF, ATG, Anti-CD154 mAb	salviae miltiorrhizae	methylprednisolone	326	pulmonary infection
ZHQ04	GTKO	TAC, MMF, ATG, Anti-CD154 mAb, CVF	salviae miltiorrhizae	methylprednisolone	116	CC

Lentiviral transfection

The TFPI sequence was inserted into the pGMLV-PA5 expression vector. Cells were seeded at 2×10^4 cells/well in 24-well plates and incubated at 37 °C for 24 hours. The medium was removed and replaced with 1 ml DMEM containing 2×10^5 (SV40T) or 5×10^5 (TFPI) lentiviral particles per well. SV40T transfection was screened using 2 ng/ml puromycin.

Mouse activation of coagulation model

The mouse activation model was described by Sparkenbaugh et al. [23]. In total, 35 µM/kg haeme (Sigma Aldrich, St. Louis, MO) was injected into mice to induce an activation of coagulation. Three hours later, pig BMSCs, lentiviral vector transfected pig BMSCs or human TFPI-transfected pig BMSCs were injected into mice via the tail vein (2.5×10^8 / kg cells were used). A recalcification assay for mouse plasma was then performed. Mice injected with normal saline were used as controls.

Western blotting

Blots were immunostained with anti-human TFPI and an appropriate secondary antibody conjugated to horseradish peroxidase. Chemiluminescence was used to visualise labelled proteins.

Statistics

The statistical tests were performed using SPSS 19 (IBM, USA). All of the results are presented as the mean $\pm$ standard deviation. Differences between two groups were determined with the t-test and were considered significant at $P < 0.05$.

Results

Recipient TF, rather than donor TF, is activated early after liver xenotransplantation

GTKO pig-to-Tibetan macaque heterotopic auxiliary liver transplantation was used to study the origin of activated TF. The immunosuppression, anticoagulation anti-inflammatory agents and cause of death/euthanasia were listed in Table 1. In the presence of a donor liver and recipient liver, all of the recipients developed consumptive coagulopathy within 2 hours after reperfusion, as observed by the presence of thrombocytopenia, thrombin-antithrombin (TAT) complexes, and fibrin. Platelet counts fell from 325 ± 24 to $122 \pm 20 \times 10^9$ /l at 2 hours after transplantation and continued to decrease rapidly. TAT complexes increased rapidly from 9 ± 1 to 186 ± 32 mg/l at 2 hours after reperfusion and remained high until 48 hours after transplantation. The fibrinogen level decreased continuously throughout the experiment (Fig. 2A).

Immunohistochemistry was performed to detect TF expression in recipient and donor livers. At both 2 and 48 hours after transplantation, primate TF was negative in donor liver but positive in recipient liver, and pig TF was negative in both (Fig. 2B). RT-PCR showed that TF mRNA in both donor liver and recipient liver was significantly upregulated at 2 hours, 48 hours and at the time of euthanasia (Fig. 3A) compared to before transplantation. However, it was calculated that from pre-transplantation to 2 hours post-transplantation,

Fig. 1. Heterotopic auxiliary liver transplantation from GTKO pigs to Tibetan macaques. (A) A liver graft harvested from a pig donor. (B) Surgical steps. i: splenectomy, ii: interruption of the left renal vein, iii: liver graft, iv: reconstruction of graft circulation, v: magnified image of vascular anastomosis, vi: biliary drainage of the donor liver. For vessels, red = artery, blue = vein, purple = portal vein. (C) Computed tomography angiogram showing good blood supply 24 hours after transplantation.

pig TF mRNA was elevated 1.42 ± 0.15 -fold in donor liver, and primate TF was elevated 2.10 ± 0.24 -fold in recipient liver ($t=4.162$, $P=0.014$, $n=3$, Fig. 3B). A significant up regulation was also observed in recipient heart and kidney (heart: 81.4 ± 31.4 vs. 16.7 ± 6.6 , $t=2.852$, $P=0.046$, $n=3$; kidney: 44.1 ± 10.6 vs. 11.6 ± 6.5 , $t=3.694$, $P=0.021$, $n=3$, Fig. 3C). Recalcification assays showed that the clotting time with recipient PBMCs after transplantation (190 ± 49 seconds) was significantly shorter than before transplantation (627 ± 53 seconds, $t=10.49$, $P=0.001$, $n=3$, Fig. 3D).

Pig TFPI does not block the human TF pathway as effectively as human TFPI

To determine the effect of pig and human TFPI on human TF, pig AECs and human monocytes were co-cultured for 8 hours. Human monocytes were human TF positive after co-incubation (Fig. 4A). The monocytes were separated from human plasma and used for the recalcification clotting assay. Human monocytes were pre-treated with recombinant human TFPI, pig TFPI, or anti-human TF. Pig TFPI did not inhibit clotting as efficiently as human TFPI (239 ± 26 vs. 301 ± 14 seconds, respectively, $t=2.976$, $P=0.041$, $n=3$, Fig. 4B). In the TFPI activity assay, pig TFPI did not inhibit human TF as efficiently as human TFPI (Fig. 4C).

Pig TFPI Kunitz domain 2 binds human FXa effectively, but Kunitz domain 1 does not inhibit human TF/FVIIa

TFPI inhibits the activation of TF in two sequential steps. First, Kunitz domain 2 of TFPI binds FXa. Second, Kunitz domain 1 of TFPI binds and inhibits the TF/FVIIa complex. We compared TFPI sequences in Wu Zhishan miniature pigs, large white pigs, and humans. We found that the two pig strains had the same coding sequence, and it was 96.6% similar to the human coding sequence. To study the exact roles of the three Kunitz domains of pig TFPI in species incompatibility, we next constructed three chimeric proteins using individual human

Fig. 2. TF in donor liver activated early after liver xenotransplantation. (A) TAT complexes, fibrinogen, and platelet counts 24 hours before and after transplantation ($t = 0$ hours). (B) TF immunohistochemistry of human and pig TF in donor and recipient livers before, 2 hours after, and 48 hours after transplantation.

Kunitz domains to replace the corresponding pig domains. These chimeric proteins, in which human Kunitz domain 1, 2, or 3 replaced the corresponding pig Kunitz domain, were named pig TFPI_{hK1}, TFPI_{hK2}, and TFPI_{hK3}, respectively (Fig. 5A). All of the chimeric proteins were constructed using standard molecular biological techniques and were verified by western blotting (Fig. 5B).

Pig TFPI_{hK1}, TFPI_{hK2}, and TFPI_{hK3}, human TFPI, and pig TFPI were analysed for their ability to bind FXa and inhibit TF/FVIIa. In the FXa binding assay, in which thrombin generation was initiated directly by FXa, different sources of TFPI bound FXa similarly (Fig. 6A). However, AFM measurements showed that the intermolecular force between pig TFPI and human FXa (69 pN) was weaker than that between human TFPI and human FXa (91 pN, Fig. 6B). In the TFPI activity assay, the inhibition of human TF/FVIIa by pig TFPI_{hK1} was similar to that of human TFPI, whereas pig TFPI_{hK2}, TFPI_{hK3}, and pig TFPI all had reduced inhibition (Fig. 6C). In the human plasma recalcification assay, pig TFPI_{hK1} inhibited TF/FVIIa well (290 ± 14 seconds) and was similar to recombinant human TFPI (298 ± 24 seconds, $t = 0.499$, $P = 0.644$, $n = 3$), and both were significant compared with pig TFPI-treated human monocytes (236 ± 24 seconds; human TFPI: $t = 3.164$, $P = 0.034$, $n = 3$; pig TFPI_{hK1}: $t = 3.366$, $P = 0.028$, $n = 3$). Pig TFPI_{hK2}

Fig. 3. Recipient TF activated after xenotransplantation (A) TF mRNA in the recipient (primate TF) and donor (pig TF) livers at different times after xenotransplantation (closed bar: recipient liver; open bar: donor liver, TX = transplantation). (B) Fold increase in TF mRNA in recipient (primate TF) and donor (pig TF) livers 2 hours after transplantation. (C) TF mRNA in native heart and kidney after xenotransplantation (Tibetan macaques without surgery were used as controls). (D) The recalcification time of normal human plasma and recipient monocytes before and 2 hours after transplantation. (* $p < 0.05$ vs. before transplantation or control, ** $p < 0.05$ vs. donor liver).

and Pig TFPI_{hK3} demonstrated a weaker inhibition of TF/FVIIa than did human TFPI (TFPI_{hK2}: 246 ± 19 seconds, $t=2.942$, $P=0.042$, $n=3$; TFPI_{hK3}: 240 ± 15 seconds, $t=3.550$, $P=0.024$, $n=3$, Fig. 6D).

To confirm the effect of different Kunitz domains on the species incompatibility of pig TFPI, the TFPI activity assay and recalcification assay were repeated with TFPI using pig Kunitz domains to replace the corresponding human domains (human TFPI_{pK1}, human TFPI_{pK2} and human TFPI_{pK3}). In the TFPI activity assay, human TFPI_{pK2} and human TFPI_{pK3} inhibited the activation of TF/FVIIa well and prolonged clotting time, similar to human TFPI whereas human TFPI_{pK1} had reduced inhibition (Fig. 6E). In the human plasma recalcification assay, both human TFPI_{pK2} (290 ± 15 seconds) and human TFPI_{pK3} (300 ± 36 seconds) inhibited TF/FVIIa well and were similar to recombinant human TFPI (292 ± 22 seconds, human TFPI_{pK2}: $t=0.130$, $P=0.903$, $n=3$; human TFPI_{pK3}: $t=0.365$, $P=0.734$, $n=3$), and both were significant compared with pig TFPI-treated human monocytes (226 ± 31 seconds; human TFPI_{pK2}: $t=3.219$, $P=0.032$, $n=3$; human TFPI_{pK3}: $t=2.924$, $P=0.043$, $n=3$). Human TFPI_{pK1} (235 ± 25 seconds) demonstrated a weaker inhibition of TF/FVIIa than human TFPI ($t=2.965$, $P=0.041$, $n=3$) but was similar to pig TFPI ($t=0.391$, $P=0.715$, $n=3$, Fig. 6F).

Immortalised pig BMSCs have the same characteristics as primary cells

Based on the above results, recipient TF is important in coagulation dysregulation after pig-to-primate xenotransplantation but is not effectively inhibited by donor TFPI. Thus, we developed pig BMSCs that overexpress human TFPI and can be co-transplanted in future pig-to-primate xenotransplantation. First, primary pig BMSCs were obtained from GTKO pig bone marrow and immortalised by transfection with SV40T. SV40T/ β -actin mRNA in SV40T-transfected BMSCs (1.067 ± 0.193) was much higher than in primary or lentiviral vector transfected cells (primary cells: 0.063 ± 0.042 , $t=11.370$, $P < 0.001$, $n=3$; lentiviral vector transfected cells: 0.075 ± 0.038 , $t=11.280$, $P < 0.001$, $n=3$; Fig. 7A). Immortalised

Fig. 4. Pig TFPI does not inhibit the human TF pathway as effectively as human TFPI. (A) Monocytes were TF positive after co-incubation with pig AECs. (B) The clotting times of recalcified human plasma in the presence of control monocytes or monocytes pre-treated with recombinant human TFPI, recombinant pig TFPI, or anti-human TF antibody. (C) Measurement of endogenous tissue factor activity for recombinant human TFPI, recombinant pig TFPI, and no TFPI control.

Fig. 5. Comparison of the TFPI coding sequences and structure of chimeric TFPI proteins. (A) Chimeric pig TFPI proteins were constructed using human Kunitz domain sequences (hK1, hK2, hK3) to replace the corresponding pig sequences (pK1, pK2, pK3). (B) Chimeric proteins, recombinant human TFPI, and recombinant pig TFPI were verified by western blotting with an anti-His antibody.

BMSCs showed a strong ability to proliferate (Fig. 7B), and no morphological differences were observed before or after SV40T transfection using light microscopy (Fig. 7C).

To further demonstrate that immortalised BMSCs had the same phenotype as primary BMSCs, the cells were analysed for the expression of CD29, CD90, CD105, CD80, CD34,

Fig. 6. Pig TFPI Kunitz domain 2 binds human FXa, but pig Kunitz domain 1 does not effectively inhibit human TF/FVIIa. (A) Pig TFPI_{hK1}, TFPI_{hK2}, TFPI_{hK3}, human TFPI, and pig TFPI have a similar capacity to bind human FXa. Left: TFPI inhibition of thrombin generation by human FXa. Right: The efficiency of inhibition compared with the no TFPI control. (B) Intermolecular forces between human FXa and human TFPI (left) or pig TFPI (right). $F_{\text{pig TFPI}} = 69$ pN, $F_{\text{human TFPI}} = 91$ pN. (C) A TFPI activity assay for human TFPI, pig TFPI_{hK1}, pig TFPI_{hK2}, pig TFPI_{hK3}, and pig TFPI. (D) Recalcification clotting assay for monocytes pre-incubated with pig TFPI, pig TFPI_{hK1}, pig TFPI_{hK2}, pig TFPI_{hK3}, and human TFPI. (E) A TFPI activity assay for human TFPI, human TFPI_{pK1}, human TFPI_{pK2}, human TFPI_{pK3}, and pig TFPI. (F) A recalcification clotting assay for monocytes pre-incubated with pig TFPI, human TFPI_{pK1}, human TFPI_{pK2}, human TFPI_{pK3}, and human TFPI.

Fig. 7. The characteristics of pig BMSCs after immortalisation by SV40T transfection. (A) RT-PCR amplification of SV40T mRNA from primary cells, lentiviral vector-transfected cells, and immortalised cells. (B) MTT proliferation assay. (C) Morphological characteristics of primary cells (left) and immortalised cells (right) as viewed by light microscopy (100×). (D) Flow cytometry of immortalised cells. (E) Differentiation assays of primary and immortalised cells. Left: oil red-O staining after adipogenic differentiation culture. Right: silver nitrate staining after osteogenic differentiation culture (200×).

and CD45 by flow cytometry. Transfected cells were positive for CD29, CD90, and CD105 and negative for CD80, CD34, and CD45 (Fig. 7D), which was the same profile described previously for primary cells [24]. In addition, immortalised BMSCs were cultured in MSC osteogenic differentiation medium or adipogenic differentiation medium for 28 days to determine the effect of SV40T transfection on BMSC differentiation. BMSCs were then stained with silver nitrate (osteogenic culture) or oil red-O (adipogenic culture). SV40T-transfected cells showed the same differentiation ability as primary cells (Fig. 7E).

Lentivirus-mediated transfection enables pig BMSCs to express human TFPI

After pig BMSCs were immortalised, a lentiviral vector was used to transfect human TFPI into pig BMSCs. The human TFPI sequence was inserted into pGMLV-PA5 to generate the recombinant lentivirus. Human TFPI mRNA expression was detected with real-time PCR from passage 0 (before transfection) to passage 7. A significant, stable rise in TFPI mRNA was observed after transfection (Fig. 8A). Western blotting (Fig. 8B) and immunohistochemistry (Fig. 8C) were used to confirm the up regulation of TFPI after transfection.

Human TFPI-transfected pig BMSCs inhibit the TF pathway in vitro

The TFPI activity assay and human plasma recalcification assay were used to examine the efficacy of human TFPI in transfected pig BMSCs. The inhibition of TF activation with human TFPI-transfected BMSCs was similar to that using recombinant human TFPI (Fig. 9A). Furthermore, pretreatment with anti-human TFPI restored TF activation to a level that

Fig. 8. Human TFPI is expressed on pig BMSCs after lentiviral transfection. (A) Real-time PCR of TFPI mRNA in pig BMSCs before (generation 0) and after (generations 1–7) human TFPI transfection. (B) Western blot assay showing strong up regulation of human TFPI in transfected pig BMSCs. (C) Strong surface expression of human TFPI was detected after transfection (right) compared with control (left) (200 $\times$). (The control in all of the panels is SV40T-transfected, TFPI-untransfected pig BMSCs.) (* $p < 0.01$ vs. before transfection).

was similar to vector-transfected and untransfected BMSCs. Human monocytes were first co-incubated with pig AECs for 8 hours, then isolated and co-incubated with pig BMSCs for another 4 hours. Compared with lentiviral vector-transfected pig BMSCs, a significant inhibition of clotting was observed using human TFPI-transfected pig BMSCs (182 ± 21 vs. 293 ± 12 seconds, respectively, $t = 6.609$, $P = 0.003$, $n = 3$, Fig. 9B). The inhibition was significantly weaker when human TFPI-transfected pig BMSCs were pretreated with anti-human TFPI (215 ± 14 seconds, $t = 5.847$, $P = 0.004$, $n = 3$).

To evaluate the effect of the human TFPI-transfected pig BMSCs on clotting, a mouse model was used, in which haeme was employed to induce the expression of TF. After the injection of haeme, a reduction in the clotting time (74 ± 19 seconds) was observed in mice compared to mice without a haeme injection (120 ± 15 seconds, $t = 3.219$, $P = 0.030$, $n = 3$). Then, human TFPI-transfected pig BMSCs were injected via the tail vein of the mice. In the mouse plasma recalcification assay, a prolongation of clotting time was observed in mice injected with the transgenic pig BMSCs ($t = 3.213$, $P = 0.033$, $n = 3$, compared with haeme-injected mice) but not in mice injected with untreated pig BMSCs (80 ± 11 seconds), normal saline (76 ± 17 seconds) or lentiviral vector (72 ± 19 seconds) (normal saline: $t = 0.136$, $P = 0.899$, $n = 3$; pig BMSCs: $t = 0.473$, $P = 0.661$, $n = 3$; lentiviral vector: $t = 0.129$, $P = 0.904$, $n = 3$, all compared with haeme-injected mice, Fig. 9C).

Discussion

Dysregulated coagulation is a major cause of graft loss in xenotransplantation [25]. Coagulation dysregulation after transplantation is associated with the activation of TF. Our initial observations in this study suggest that activated TF after pig-to-primate xenotransplantation originates from the recipient. When we next examined why recipient TF was not inhibited efficiently, we identified a species incompatibility between human TF and pig TFPI, with Kunitz domain 1 playing the most important role in the incompatibility. As a first step in solving this incompatibility, we developed pig BMSCs that express human TFPI and demonstrated their ability to inhibit human TF *in vitro*.

Previous studies showed that TF activated by the immune response (IR) in donor livers is the main cause of coagulation dysregulation [5, 7, 8]. Ekser et al. [8] showed that recipient TF on recipient monocytes is activated earlier than TF on donor livers after liver xenotransplantation. In our current study, we established a new liver xenotransplantation

Fig. 9. Human TFPI-transfected pig BMSCs inhibit clotting *in vitro* and in a rodent model. (A) A TFPI activation assay with recombinant human TFPI, human TFPI-transfected pig BMSCs, human TFPI-transfected pig BMSCs pretreated with anti-human TFPI, lentiviral vector-transfected immortalised pig BMSCs, and untreated immortalised pig BMSCs. (B) A recalcification assay. Human monocytes were co-incubated with pig AECs (pAECs), and then co-incubated with or without human TFPI-transfected pig BMSCs (pBMSCs) or lentiviral vector-transfected immortalised BMSCs. The activity was compared to monocytes treated with pAECs followed by recombinant human TFPI. Closed bar: human TFPI-transfected pig BMSCs pretreated with anti-human TFPI. (C) The recalcification of mice that, without treatment, induced TF activation by injecting TF, and the TF-activated mice injected with normal saline, lentiviral vector and human TFPI-transfected pig BMSCs.

model to investigate the roles of recipient and donor livers. The recipient in this study showed nearly the same changes in coagulation as when the recipient liver is removed [5, 17]. Our comparison of recipient organs and donor livers provides more evidence that recipient TF is activated earlier after transplantation than TF from the donor liver. Recipient TF activation and the occurrence of CC were synchronised. Furthermore, we found no evidence that the activation of donor TF was correlated with the initiation of coagulation dysregulation.

These results were consistent with those of Lin et al. [11]. In both studies, the native organs were more likely to express TF than donor organs after xenotransplantation. Moreover, in Lin's study, the activation of platelets and its "cross-talk" with leukocytes seems to play an important role in the induction of CC. Likewise, in our study, the activation of monocytes was also observed in recipients when CC occurred. It seems that TF activation after xenotransplantation is a systemic response in recipients. In both studies, recipient

TF, rather than donor TF, was activated once CC occurred. It is a reasonable speculation that recipient TF may correlate with CC after xenotransplantation. Furthermore, our study demonstrates that inhibiting the activation of recipient TF may provide a meaningful method for controlling coagulation dysregulation.

Our study also suggests that failure to inhibit the activation of recipient TF was due to species incompatibility of pig TFPI and that Kunitz domain 1 plays the most important role in this incompatibility. Pig TFPI Kunitz domain 2 also affected the inhibition of human TF but not as significantly as domain 1, possibly due to the difference in the binding strength between FXa and human versus pig TFPI. We found no evidence that the Kunitz domain 3 plays a role in species incompatibility by binding to the cell surface GPI anchor through co-receptors. As the co-receptors for TFPI binding to the cell surface are not entirely known [10], the role of their interaction with TFPI in species incompatibility requires further study.

Other groups have shown different results in studies of the incompatibility of pig TFPI. Kopp [15] showed that pig TFPI on pig AECs fails to bind human FXa and thus fails to inhibit human TF/FVIIa. Cultured pig AECs may express considerably lower levels of firmly attached TFPI on their surface than human AECs [16]. Lee showed that recombinant pig TFPI binds human FXa and efficiently inhibits human TF/FVIIa, perhaps because of their use of the FLAG tag as an indirect way to test TFPI expression on the cell surface. The differences in the assays used may have caused the contrasts between the studies. Because TFPI is glycosylated in the ER and usually functions on the cell surface, the use of cell free and non-glycosylated TFPI in the TFPI assay has the potential to be a factor that influences the results. Regardless, the overexpression of human TFPI here contributed to the regulation of coagulation, as shown with cardiac xenotransplantation [26].

What is the best way to overexpress human TFPI in pig-to-primate liver xenotransplantation recipients? The injection of exogenous recombinant TFPI may not be efficient because free TFPI in the plasma can be degraded by a variety of proteinases [12], and the co-receptor binds to TFPI in the endoplasmic reticulum/Golgi only inside cells [13]. Another possible option is breeding donor pigs transfected with human TFPI. However, transgenic pigs with other genes, such as CD46, CD55, CD59, and thrombomodulin, have also shown a potential to prolong survival following xenotransplantation [27–30]. In such cases, we developed donor human TFPI-transfected BMSCs as an alternative way to overexpress human TFPI in xenotransplantation when pigs transfected with other genes (without TFPI) were used as donors. BMSCs were chosen to be the carriers of the genes due to their adequate availability, easy accessibility, rapid proliferation, and successful integration and immunological tolerance in the host tissue [24]. Our human TFPI-transfected pig BMSCs showed high-efficiency binding of human FXa and inhibited TF/FVIIa *in vitro* and in a rodent model, demonstrating that they are effective gene carriers. Jie et al. [31] showed that the survival of pig-to-rhesus xenografts is prolonged by prior donor bone marrow transplantation. Further studies showed that BMSCs can induce immunosuppression by inhibiting the proliferation of T cells and the secretion of immunosuppressive factors such as interleukin-10 and transforming growth factor β [32–36]. This result has been further demonstrated in xenotransplantation models using donor BMSCs co-transplanted with the liver or cellular nerve grafts [32, 36]. Obviously, the movement, attachment, and differentiation of BMSCs and the expression of TFPI and its effect on the coagulation need to be evaluated *in vivo*. Moreover, the use of GTKO pigs expressing human complement and coagulation regulatory proteins would be more contemporary as they are able to eliminate “noise” caused by other pathways. These types of experiments would be considered in our future studies.

In summary, early activation of recipient TF is responsible for coagulation dysregulation after liver transplantation. The incompatibility between pig TFPI and human TF leads to the inefficient inhibition of recipient TF. The overexpression of human TFPI by pig BMSCs inhibits clotting *in vitro*. Our results offer a predictable approach to addressing the problem of coagulation dysregulation after xenotransplantation. This approach will be investigated in our *in vivo* xenotransplantation model.

Acknowledgements

These experiments were supported by the National Basic Research Program of China (973 Program; 2015CB554100), the National Natural Science Foundation (81270549 and 81300361), the Xijing Hospital disciplines boosting project (XJZT12M09), the Xijing Hospital disciplines boosting project (XJZT13Z01), the Science and Technology Research and Development Program of Shaanxi province (2013K12-18-02) and the National High Technology Research and Development Program (863 Program; 2012AA021005).

Disclosure Statement

There are no conflicts of interest in this work.

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