

The role of donor versus recipient tissue factor in coagulopathy during pig-to-primate xenotransplantation

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ABSTRACT

The increasing demand for organs has stimulated the necessity in xenotransplantation, which promises an unlimited supply of organs for clinical use. However, coagulopathy of xenografts remains a major hurdle to successful pig-to-primate xenotransplantation. The ability to generate pigs expressing a human complement-regulatory protein (hCRP) and/or pigs in which the $\alpha 1,3$ -Galactosyltransferase gene has been knocked-out (GT-KO) has largely overcome the barrier of hyperacute rejection (HAR) of a pig organ transplanted into a primate. However, acute humoral xenograft rejection (AHXR), presenting as microvascular thrombosis with/without consumptive coagulopathy (CC), ensues and results in graft failure.

The causes of coagulopathy were believed to be humoral response-dependent. Xenoreactive antibodies (Abs) and activation of complement provoke porcine endothelial cells (ECs) from an anticoagulant to a procoagulant phenotype. In this study, I demonstrated that recipient platelets and monocytes were activated to express tissue factor (TF), an initiator of the coagulation cascade, after incubation with porcine ECs through humoral immune response-independent process. These observations were mirrored in the animal studies. Kidneys or livers from GT-KO pigs that express a hCRP transplanted into nonhuman primates were not susceptible to HAR. Nevertheless, most recipients developed CC, even when the grafts were still functioning. Activation of graft ECs and the measurable immune response were minimal. TF expression on recipient platelets plays a pivotal role in initiating CC. Therefore, understanding the interactions between porcine ECs and primate platelets may be crucial to prevent coagulopathy. On the other hand, the generation of GT-KO pigs has directed interest to the role of anti-nonGal Abs in intravascular thrombosis.

My study revealed that anti-nonGal Abs activated porcine ECs to express TF, independent of complement activation.

I also demonstrated that anti-P-selectin and vWF Abs and some anti-platelet agents, such as clopidogrel and eptifibatide, prevented TF expression on platelets after incubation with porcine ECs. Porcine ECs from pigs that expressed tissue factor pathway inhibitor (TFPI) were resistant to the activation induced by primate serum even with high titre anti-nonGal Abs. Atorvastatin not only inhibited this activation of platelets but also prevented the activation of porcine ECs induced by primate serum.

Coagulopathy is increasingly recognized as barriers to successful xenotransplantation, many mechanisms of which are not associated with humoral immune response. Further manipulation of the immune response alone, with the risk of inducing infection and other complications, does not appear likely to resolve the challenge of xenograft coagulopathy. My results provide evidence for further genetic manipulation or systemic pharmaceutical treatment to prevent coagulopathy seen after pig-primate xenotransplantation.

DECLARATION

1. The thesis comprises only my original work towards the PhD, except where indicated.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliography and appendices.

PREFACE

The work was performed in Imperial College London (Chapter 3) and the University of Pittsburgh Medical Centre (Chapters 4,5,6,7).

All work in this thesis is my original research except:

1. The aortic patch and kidney xenotransplantations were organized by Dr Cooper and Dr Ezzelarab.
2. Liver xenotransplantations were organized by Dr Cooper and Dr Eksler.

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PRESENTATIONS

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6. **Chih Che Lin**, Mohamed Ezzelarab, Hidetaka Hara, Cassandra Long, David Ayares, Anthony Dorling, David K.C. Cooper. Tissue Factor Expression on Recipient Platelets Predicts the Development of Consumptive Coagulopathy in Pig-to-Primate Organ. Platelets International Symposium, Boston, 2008.
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9. **Chih Che Lin**, Burcin Ekser, Bruno Gridelli, Cassandra Long, Gabriel Echeverri, Mohamed Ezzelarab, David Ayares, Anthony Dorling, David K.C. Cooper. Tissue factor expression and activity on recipient platelets and PBMCs is the key factor in coagulation dysfunction in pig-to-primate liver transplantation. Congress of International Society on Thrombosis and Haemostasis, Boston, 2009.

PUBLICATIONS

1. **Lin CC**, Chen D, McVey JH, Cooper DK, Dorling A. Expression of tissue factor and initiation of clotting by human platelets and monocytes after incubation with porcine endothelial cells. *Transplantation*. 2008;86(5):702-709.
2. Ezzelarab M, Garcia B, Azimzadeh A, Sun H, **Lin CC**, Hara H, Kelishadi S, Zhang T, Lin YJ, Tai HC, Wagner R, Thacker J, Murase N, McCurry K, Barth RN, Ayares D, Pierson RN, Cooper DK. The innate immune response and activation of coagulation in α 1,3-galactosyltransferase gene-knockout xenograft recipients. *Transplantation*. 2009 ;87(6):805-12.
3. **Lin CC**, Cooper DK, Dorling A. Coagulation dysregulation as a barrier to xenotransplantation in the primate. *Transpl Immunol*. 2009; 21(2):95-80.
4. Ekser B, Long C, Echeverri GJ, Hara H, Ezzelarab M, **Lin CC**, de Vera ME, Wagner R, Klein E, Wolf RF, Ayares D, Cooper DK, Gridelli B. Impact of Thrombocytopenia on Survival of Baboons with Genetically Modified Pig Liver Transplants: Clinical Relevance. *Am J Transplant*. 2009 (accepted)
5. **Lin CC**, Ezzelarab M, Hara H, Long C, Dorling A, Cooper DK. Atorvastatin or transgenic expression of TFPI inhibits coagulation initiated by anti-nonGal IgG binding to porcine aortic endothelial cells. (submitted)
6. **Lin CC**, Ezzelarab M, Shapiro S, Ekser B, Long C, Hara H, Echevirri G, Torres C, Watanabe H, Ayares D, Dorling A, Cooper DK. Tissue factor expression on recipient platelets initiates consumptive coagulopathy in pig-to-primate kidney transplantation. (submitted)
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ABBREVIATIONS

Abbreviation	Full name
(+/-)	Homozygous for the transgene
Ab	Antibody
ACK	Ammonium chloride-potassium
ADP	Adenosine diphosphate
AHXR	Acute humoral xenograft rejection
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AVR	Acute vascular rejection
BS	Baboon serum
cAMP	Cyclic adenine monophosphate
CLK1	Cdc2-like kinase
CMV	Cytomegalovirus
CRP	Complement regulatory protein
CVF	Cobra venom factor
DAF	Decay-accelerating factor (DAF, CD55)
DAPI	4',6-diamidino-2-phenylindole
DIC	Disseminated intravascular coagulation
ECs	Endothelial cells
ecto-NAPDases	Ecto-nucleoside ATP diphosphohydrolases
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate

FVII	Factor VII
Gal	Gal α 1,3 gal β 1,4 GlcNac
GT-KO	α 1,3-galactosyltransferase knock-out
HAR	Hyperacute rejection
HI	Heat-inactivated
HP	Human plasma
IBMIR	Instant blood-mediated inflammatory reaction
ICAM 1	Inter-cellular adhesion molecule 1
Ig	Immunoglobulin
iGb3	Isoglobotrihexosylceramide
LDH	Lactate dehydrogenase
MAC	Membrane attack complex
MCP	Membrane cofactor protein (CD46)
MFI	Mean fluorescence intensity
NF- κ B	Nuclear factor- κ B
PAECs	Porcine aortic endothelial cells
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
TBS	Tris buffered saline
TF	Tissue factor

TFPI	Tissue factor pathway inhibitor
TM	Thrombotic microangiopathy
TNF	Tumor necrosis factor
TRITC	Tetramethyl rhodamine isothiocyanate
VCAM-1	Vascular cellular adhesion molecule 1
vWF	von Willebrand factor
WT	Wild-type
XNA	Xenoreactive antibodies
α 1,3 GT	α 1,3-galactosyltransferase

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CHAPTER 1

INTRODUCTION

1.1 ORGAN SHORTAGE

1.1.1 Progress of organ transplantation

Since the first kidney transplantation was performed in 1954 between identical twins (Merrill et al., 1956), organ transplantation has emerged as a definite treatment for many end-stage organ diseases. In the past decades, the 5-year survival rates of patients increased to over 80% in kidney, and 70% in liver and heart transplantation in the USA (Sayegh and Carpenter, 2004). At the end of 2002 in the USA, 150,000 people lived with functioning solid-organ allografts, up from 62,000 in 1993 (www.ustransplant.org).

1.1.2 Organ demand and shortage

The success of organ transplantation has dramatically increased the discrepancy between organ demand and supply. In the USA, the number of patients on the waiting list for transplantation increased from 37,252 to 98,236 between 1993 and 2007. At the same time, the number of donors just increased from 14,638 to 28,231 (www.ustransplant.org). In Asian countries, the gap is more critical because the organ donation rate is much lower than that in western countries (de Villa et al., 2003). The ever-growing deficiency of donors has stimulated the necessity of alternative organ resources, such as living donors, marginal donors, or organs from other species.

1.1.3 Expanded organs (marginal organs)

One innovative way to increase the number of deceased donors is to use the organs from expanded criteria donors or donors after cardiac death. However, these transplants are often associated with inferior outcomes and higher costs. Moreover, although a great effort has been devoted to maintain the organs after cardiac arrest, organs from donors after cardiac death still remain scarce (D'Alessandro et al., 2008). Another way is to use deceased donors with certain types of infections, such as hepatitis B and C. This also puts the recipients in a risk of overwhelming infection and even mortality. The expanded use of marginal organs raises potential ethical considerations about appropriate recipient selection and the high cost of medical care (Pomfret et al., 2008).

1.1.3.1 Living donors

The use of kidneys from living donors significantly shortens the waiting time and provides better patient and graft survivals, compared to deceased donor transplantation (Davis and Delmonico, 2005). In the USA, the number of live kidney donors has surpassed the number of deceased donors. However, in the areas where deceased donors are few, most centres perform kidney transplantation from live donors. Similarly, living-donor liver transplantation has rooted from Asian countries. In experienced centres; liver transplantation from live donors reduces the waitlist mortality. Post-transplant patient and graft survivals are equivalent to those from deceased donors (Chen et al., 2003).

However, living donor donation has entailed a risk to healthy donors. The mortality rate of hepatic lobe donor for liver transplantation is around 0.15-0.2% and the overall complication rate is up to 38% although the reasons are not definitely related to donor

surgery (Olthoff et al., 2005; Trotter et al., 2006). The overall percentage of live donor liver transplants peaked at 10% of the total number of liver transplants performed in 2001. Since then, the percentages have reduced to 5% of the total, about 320 each year in the USA (www.ustransplant.com).

1.1.4 The necessity of xenotransplantation

No matter how great an effort has been dedicated, the gap between demand and supply of organs is burgeoning and is predicted to remain so. The use of animal organs, xenotransplantation, if the immunologic barriers and associated problems could be solved, may provide an unlimited alternative to allografts. In the last century, some attempts were made to transplant kidneys or livers from primates or non-primate mammals into human. The longest survivals of kidney, heart and liver from primate donors were 9 months, 20 days, and 70 days, respectively (Appel et al., 2002). However, the results from non-primate organs were dismal, surviving hours rather than days.

1.2 PIGS AS SOURCES OF DONORS

From the immunologic perspective, non-human primates would be preferable organ donors. However, many of them are endangered species, and the time and expense of breeding these animals are also prohibitive. Therefore, the UK Department of Health Advisory Group on the Ethics of Xenotransplantation recommended that “it would be ethically unacceptable to use primates as source animals for xenotransplantation”. Secondly, concerns about the transmission of infectious agents from nonhuman primates to humans have been raised, particularly since most non-human primates are either wild-

caught or have been housed under colony conditions for relatively few generations. Furthermore, there is no experience in generating genetically-modified primate animals. Instead of primates, pigs are thought the most suitable animals as a source of xenografts for human.

1.2.1 Physiology of pig and human

Pigs emerge as the most suitable source of xenograft organs. They are easily bred with sexual maturity within 9 months, gestation of 3.5 months, and litters of 6-16 piglets. Furthermore, the physiology and anatomy of organs in adult pigs are similar to adult human with life expectancy of approximate 30 years. The hematological and chemistry profiles are compatible with those in adult humans and non-human primates (Figure 1.2-1).

Parameters	Baboon	Pigs
Red blood cells (RBC) ($\times 10^6/\mu\text{l}$)	5.1 $\pm$ 0.4	5.1 $\pm$ 0.8
White blood cells (WBC) ($\times 10^3/\mu\text{l}$)	8.5 $\pm$ 4.4	15.0 $\pm$ 7.1
Hemoglobin (Hb) (mg%)	12.5 $\pm$ 1.0	9.4 $\pm$ 1.1
Hematocrit (Hb) (%)	38.0 $\pm$ 3.3	26.6 $\pm$ 3.9
Platelets (PLT) ($\times 10^3/\mu\text{l}$) (U/L)	297 $\pm$ 73	291 $\pm$ 106
Alanine aminotransferase (ALT) (U/L)	34.5 $\pm$ 15	75.9 $\pm$ 19.2
Aspartate aminotransferase (AST) (U/L)	38.7 $\pm$ 15.6	42.8 $\pm$ 15.3
Gamma glutamyl transferase (GGT) (U/L)	53.5 $\pm$ 13.3	56.9 $\pm$ 42.8
Alkaline phosphatase (ALP) (U/L)	642 $\pm$ 198	140.1 $\pm$ 55.2
Total bilirubin (mg%)	0.1 $\pm$ 0	0.1 $\pm$ 0
Total protein (mg%)	6.4 $\pm$ 0.8	4.0 $\pm$ 1.0
Albumin (mg%)	6.4 $\pm$ 0.8	2.1 $\pm$ 0.9

Figure 1.2-1 Normal values of biochemistry and hematology in baboons and pigs.

The data were based on 25 baboons and pigs (provided by Dr Echeverri and Ekser).

1.2.2 Concerns of zoonosis

Since the organs from other species are directly implanted into recipients during xenotransplantation, pathogens may bypass the natural defenses of hosts, such as skin and mucosa, and become infectious to humans. Importantly, the recipients of human require receiving extensive immunosuppressive regimens and will become more susceptible to pathogens. These immuno-compromised recipients will serve the seeds for human-to-human transmission. Some porcine viruses have been identified as pathogens in human. One of the most concerns is porcine endogenous retroviruses (PERVs), which are able to infect human cell lines *in vitro* (Patience et al., 1997). Fortunately, recent retrospective studies showed no evidence of infection, expression or replication of PERVs in human recipients who had exposed to short-term extracorporeal porcine kidney or to porcine fetal islets. Recently, a study also revealed there was no evidence by infection of PERVs in clinical staff who were involved with the care of patients undergoing extracorporeal liver perfusion (Levy et al., 2007).

1.2.3 Genetic modification of pigs

In order to minimize the immune response between different species, the modification of donors that supply xenografts to human offers a unique way to solve the problems which are encountered in xenotransplantation. Advances in transgenic manipulation and cloning in swine has fulfilled the reality of genetically-modified organs from pigs. Pigs overexpressing human complement-regulatory proteins (hCRP) such as CD59, or human decay-accelerating factor (DAF, CD55) have suggested that to further modification of swine is a practical way to overcome the barriers between swine and primates.

1.2.4 Ethics of xenotransplantation

Non-human primates, such as chimpanzees, have intellectual and social natures, similar to humans. They are considered as endangered species as well. Hence, using non-human primates as donors raises ethical concerns. In contrast, from the point of view of ethics, pigs as xenograft donors raise minimal issues. There are still some other ethical issues associated with xenotransplantation. For example, whether the transfer of organs or tissues from animals is natural and whether to kill animals to benefit human beings is justified remain controversial points. Nevertheless, the shortage of organs leaves patients desperate. This demand stimulates progress in xenotransplantation from the scientific point of view.

Taken together, the aforementioned issues indicate that pigs are currently considered the most suitable sources of xenografts (Cooper et al., 1993; Elliott et al., 2000; Heneine et al., 1998) although clinical application of this technique still requires ethical, regulatory, and legal frameworks to provide guidelines and to establish a consensus.

1.3 HYPERACUTE REJECTION

Although pigs are relative ideal animals as donors of xenografts, vigorous immunologic rejection by the host's immune system ensues due to differences between species, especially the existence of natural antibodies (Abs). Various types of immunologic rejection are categorized according to their reactive times, including hyperacute rejection (HAR), acute humoral xenograft rejection (AHXR), and cellular rejection.

The most devastating immunologic response faced in pig-to primate xenotransplantation is HAR, which is induced by the binding of preformed Abs to the xenograft vascular endothelial cells (ECs) within minutes to hours (Perper and Najarian, 1967). The binding of xenoreactive Abs (XNA) to donor ECs activates the recipient's complement system, which consequently results in the deposition of C3 and terminal membrane attack complex (MAC) on ECs. This reaction leads to shape change and the formation of gaps in the absence of cytolysis (Saadi and Platt, 1995). The loss of integrity of ECs exposes the underlying subendothelial matrix, in which there are many potent ligands and substances, to trigger the coagulation cascade and platelet aggregation (Yang and Sykes, 2007). Meanwhile, the MAC activates ECs to become procoagulant because of increased cytosolic calcium and nonlytic secretion of high molecular weight multimers of von Willebrand factor (vWF) from intracellular storage granules (Hamilton et al., 1990) and the loss of natural anticoagulant properties. These changes trigger the coagulation cascade and the activation of platelets to form thrombi.

1.3.1 Loss of anticoagulant properties

In HAR, the injured ECs become thrombogenic even when they are still alive. C5b-9 proteins induce vesiculation of ECs and thereby expose a catalytic surface for the prothrombinase enzyme complex, contributing to fibrin deposition associated with immune EC injury (Hamilton et al., 1990). Moreover, activated ECs lose the capacity of natural anticoagulation (Robson et al., 2000). These anticoagulant molecules include

thrombomodulin (Ogawa et al., 1990), antithrombin, CD39, and heparan sulfate proteoglycan.

Dowregulation of thrombomodulin and antithrombin has been recognized in discordant xenografts (Blakely et al., 1994). *In vitro* studies showed that exposure of porcine aortic endothelial cells (PAECs) to human serum caused cleavage and release of proteoglycans dependent on activation of the classical complement pathway (Platt et al., 1990). A few years later, an additional thromboregulatory factor, ecto-nucleoside ATP diphosphohydrolases (ecto-NAPDases), which is also expressed by quiescent ECs, and has the capacity to degrade the extracellular inflammatory mediators, adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) is lost after ECs are activated. This change promotes platelet activation and aggregation and may be crucial for the progression of vascular injury (Robson et al., 1997). In addition, murine tissue factor pathway inhibitor (TFPI) expression was also down-regulated in cytokine-induced peritonitis models (Shimokawa et al., 2000).

These detrimental procoagulant and inflammatory environments result in microvascular thrombosis, intravascular occlusion, and ultimate graft loss within minutes to hours after reperfusion.

1.3.2 Gal antigen

The main cause of HAR results from the existence of XNA. The most important one is the Ab against the Gal α 1–3Gal β 1–4GlcNAc (Gal) epitope, which accounts for over 85%

(Cooper et al., 1993; Galili et al., 1984). The Gal epitope is a common carbohydrate structure found in many mammals, and this epitope has been reported to be expressed on glycoproteins and glycolipids of different species. In 1988, Galili investigated Gal epitope expression in mammals by immunostaining with anti-Gal Abs, and correlated this with binding of the lectin *Griffonia (Bandeiraea) simplicifolia*-IB4 (GS-IB4), which interacts with Gal residues. Many cells of non-primate mammals, prosimians and New World monkeys are readily bound by both anti-Gal Abs and the GS-IB4 lectin. The Gal epitope is synthesized by the enzyme α 1,3-galactosyltransferase (α 1,3GT) using *N*-acetyllactosamine as the sugar acceptor and uridine diphosphate-Gal as the sugar donor. However, humans, apes and Old World monkeys (monkeys of Asia and Africa) only possess the pseudogene for this enzyme; therefore, they carry anti-Gal Abs that are raised in response to antigenic stimulation by Gal epitopes expressed in gastrointestinal tract bacteria (Galili et al., 1987a; Galili et al., 1987b; Galili, 1993).

1.3.3 Depletion of xenoreactive antibodies

Since Gal Abs play a pivotal role in the pathogenesis of HAR, the activation of XNA interaction is critical to avert HAR. Removal of the natural Abs by plasmapheresis is effective in preventing HAR but entails some disadvantages, such as marked depletion of complement and coagulation proteins. The use in the peri-transplant period therefore is limited (Fischel et al., 1990). Nonspecific Ig-binding columns were designed to result in 90.5 and 86.0% reduction in total IgG and IgM, with the preservation of albumin and coagulation proteins. They resulted in prolonged xenograft function in ex-vivo perfusion systems (Kroshus et al., 1995) and averted HAR in kidney xenotransplantation models

(Leventhal et al., 1995). Gal-specific affinity columns also effectively removed anti-Gal Abs and minimized hypogammaglobulinemia in spite of the requirement of multiple absorptions (Kozlowski et al., 1998; Watts et al., 2000). However, these modalities can only be used temporarily because they require vascular access, real-time separation of blood and plasma. Importantly, the production of anti-Gal Abs continues, and near-normal levels of Abs return rapidly after cessation of immunoabsorption, which leads to graft loss.

Another approach is neutralizing anti-Gal Abs by continuous intravenous infusion of large amounts of soluble antigens, such as Gal sugar (e.g. GAS914 and TPC) (Katopodis et al., 2002; Schirmer et al., 2004; Teranishi et al., 2002). Nevertheless, despite the delayed return, anti-Gal Abs are never eliminated and graft rejection is delayed but not abrogated.

1.3.4 Genetic engineering to decrease Gal antigen expression

Since there are a large number of Gal epitopes on pig cells (35×10^6 /cell), the removal or inhibition of anti-Gal Abs in primate serum could not effectively prevent humoral rejection for a long period of time. The advantage of xenotransplantation is the opportunity to modify the donors by genetical engineering. The ideal way to eliminate the Gal antigen in pigs is to generate pigs that lack the expression of Gal epitopes by targeting the donor genome.

Pigs that express human α 1, 2-fucosyltransferase (H-transferase, HT) were generated in an attempt to reduce the expression of Gal epitopes. HT expression modifies the cell surface carbohydrate phenotype on pig cells, resulting in the expression of the universal donor O antigen and a concomitant reduction in the expression of the antigenic Gal epitopes. The significant reduction in the expression of the Gal epitopes reduced XNA reactivity, and increased resistance to complement-dependent cytotoxicity (Costa et al., 1999). Similarly, pigs that expressed the human beta-d-mannoside beta-1,4-N-acetylglucosaminyltransferase III (GnT-III) gene reduced serum reactivity to Gal epitopes by changing the configuration of glycoproteins. EC studies from the GnT-III transgenic pigs revealed a significant down-regulation in antigenicity, including the Hanganutziu-Deicher antigen, and dramatic reductions in both the complement- and natural killer cell-mediated pig cell lyses. In addition, the effect of GnT-III in down-regulating the xenoantigen expression in pig heart grafts was evidenced in an animal study (Miyagawa et al., 2001). However, this resulted in only partial reduction in epitope numbers and failed to substantially extend graft survival in primate recipients.

1.3.5 GT-KO pigs

The development of nuclear transfer technology has provided a means for locus-specific modification of large animals. It allowed for the development of pigs in which the gene of α 1,3GT is deleted. By cloning fetal fibroblast cells, which are used as nuclear donors for embryos reconstructed with enucleated pig oocytes, heterogenous GT-KO pigs have been generated in the early period of this decade (Dai et al., 2002; Lai et al., 2002). To date, homozygous GT-KO pigs are available for pig-to-primate animal studies by some groups

(Phelps et al., 2003; Sharma et al., 2003). It is worth noting that the deletion of Gal from donor organs has been recognized as a major step toward the clinical application of xenotransplantation. Transplanting hearts from GT-KO pigs into baboons, the medium survival of xenografts was extended up to over 70 days with a co-stimulatory pathway blockade-based immunosuppressive regimen (Kuwaki et al., 2005). Meanwhile, in the kidney model, the longest survival was extended up to 83 days with the tolerance-inducing regimens by co-transplantation of vasculized thymic tissue (Yamada et al., 2005).

The production of homozygous pigs is based on a disruption in the $\alpha 1,3$ GT gene, which encodes Gal epitope. The concerns have arisen regarding GT-KO pigs as to whether the Gal epitope is exclusively produced by the $\alpha 1,3$ GT gene. Isoglobotrihexosylceramide (iGb3) synthase was shown to be another candidate that synthesizes Gal epitope (Milland et al., 2006; Taylor et al., 2003). Low levels of Gal are still expressed in GT-KO mice in the form of a lipid (iGb3), which is produced by iGb3 synthase. Similarly, GT-KO cells in pigs have been demonstrated to synthesize the Gal antigen at 1 to 2% of the level of control heterozygous cells by flowcytometry (Sharma et al., 2003). Although GT-KO pigs do avert HAR and prolong graft survival, the presence of secondary enzymes, such as iGb3 synthase may possibly have implications for long-term graft survival in pig-to primate xenotransplantation using GT-KO organs.

1.3.6 Complement pathway

HAR is caused by the rapid binding of terminal complement complexes to the cell membranes of the endothelial lining of blood vessels in the donor organ (Cascalho and Platt, 2001). The complement system consists of more than 30 proteins in plasma and on cell surfaces. Complement proteins in plasma amount to more than 3g/L and constitute approximately 15% of the globulin fraction (Walport, 2001). Complement is part of the innate immune system, serves as one of the main effector mechanisms of Ab-mediated immunity, and forms the nexus of the innate and adaptive immune systems. Three biochemical pathways activate the complement system: the classical complement pathway, the alternative complement pathway, and the mannose-binding lectin pathway (Figure 1.3-1).

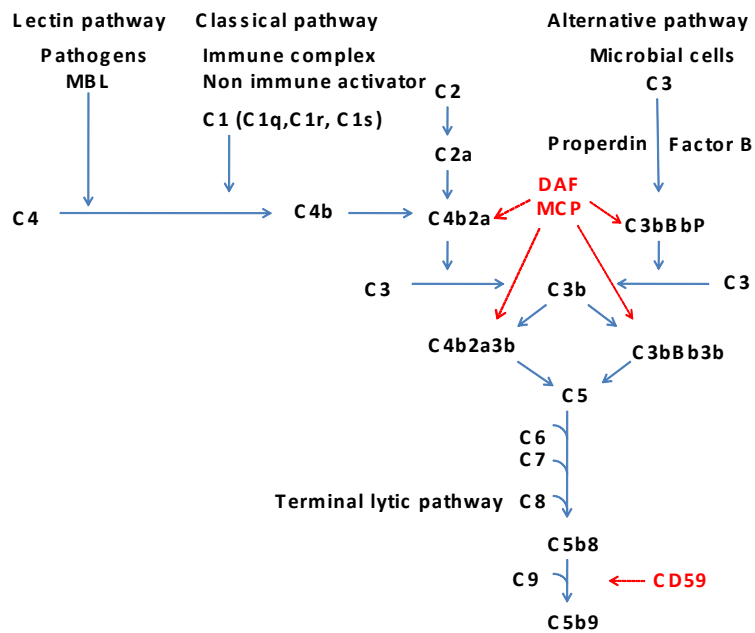


Figure 1.3-1 The pathway of complement activation and regulation.

(Adapted from Walport MJ, NEJM 2001. Blue: activation; red: regulator and inhibition; MBL: mannose-binding lectin; MCP: membrane cofactor protein; DAF: decay-accelerating factor)

The classical pathway is activated mainly by the immune response and other apoptotic cells or pathogens (Walport, 2001). Ab-mediated complement activation is initiated by the activation of C1 (which is composed of C1q, C1r and C1s) after the interaction of IgG or IgM bound to antigen epitopes. The C1q-binding capacity of human IgG subclasses is IgG3, IgG1, IgG2 and IgG4, in decreasing order. Conformational changes in C1q then allow cleavage of C1r, and activated C1r cleaves and activates C1s, which is the enzyme that activates C2 and C4.

C4 is cleaved by C1s into C4a and C4b. C4b combines with the enzymatically-active fragment C2a to form C4b-C2a, which is known as the classical pathway C3 convertase. After C3-convertase formation, the classical pathway leads to the common step of C3 activation, and cleavage to C3a and C3b. When a C3b molecule is covalently deposited in the vicinity of the C3 convertase, the form of C5 convertase C4b-C2a-C3b is generated. Cleavage of C5 then releases the bioactive peptides C5a, and C5b. C5b initiates formation of the MAC (membrane-bound C5b-C6-C7-C8-C9, denoted as C5b-C9), which causes cell lysis.

The mannose-binding lectin pathway is initiated by binding of the complex of mannose-binding lectin, which is found on the surface of some bacteria, yeasts and fungi. This

pathway activates the complement by interaction with mannose-binding lectin–associated proteases (MASP1, MASP2, MASP3 and small MAP). MASP2 acts in a fashion similar to that of C1s to lead to the formation of the C3 convertase enzyme C4b2a. MASP1 may be able to cleave C3 directly. Activation of C3 triggers the alternative pathway loop and the formation of MAC (Fujita, 2002). The activation of the alternative pathway does not require sensitization and Abs. This pathway involves the participation of C3, factor B, factor D and properdin. In this pathway, C3 undergoes spontaneous cleavage and induces binding between factor B and C3b. The C3bB complex is cleaved by factor D, releasing the factor B amino-terminal fragment (Ba), and activating the serine protease domain in the remaining C3bBb complex. The C3bBb complex can be recognized and stabilized by properdin. Dissociation of convertases leads to the irreversible deactivation of the catalytic site (Kemper and Hourcade, 2008).

1.3.7 Regulators of the complement system

Such a powerful complement system requires tight regulation; therefore, cells are resistant to complement-mediated lysis. At least three different mechanisms of regulation are known (Figure 1.3-1). CD46 was formerly called membrane cofactor protein (MCP) and exists ubiquitously, except on erythrocytes (Astier, 2008). Its primary function is to protect autologous cells from complement attack. Indeed, it binds to C3b and C4b and acts as a cofactor for the protease factor I, allowing the proteolytic cleavage of C3b and C4b (Seya and Atkinson, 1989). CD55 (DAF) inhibits formation of the C3 convertases through binding to C3b and C4b. It also binds the alternate pathway convertase C3bBb, the classical pathway convertase and C4b2a to accelerate their decay (Nicholson-Weller

and Wang, 1994). CD59 protects cells from complement-mediated lysis through binding C9, inhibiting its incorporation into the C5b-9 complex and preventing the terminal polymerization of MAC in plasma membranes (Lachmann, 1991).

1.3.8 Complement system and hyperacute rejection

Knowledge of the importance of complement and the activation and regulation of its pathway shed light on developing strategies to overcome HAR in xenotransplantation. The activation of complement resulting in HAR can be attributed to the classical and alternative pathway. Depletion of complement by cobra venom factor (CVF) (Leventhal et al., 1993) or inhibition by soluble complement receptor type 1 prevents HAR (Pruitt et al., 1994). Furthermore, C6-deficiency in rabbits and rats prevents HAR of cat and guinea pig kidneys (Zhow et al., 1990). These observations suggested activation of complement in HAR is via the classical pathway as a consequence of endothelial binding of XNA. However, other studies have demonstrated that HAR could be induced by the complement system in the absence of XNA (Johnston et al., 1992), implicating involvement of the alternative pathway of complement (Forty et al., 1992). For example, in the mouse to human *ex vivo* combination by depleting C1q or factor D, both the classical and alternative pathway was related to the activation of complement to HAR (Romanella et al., 1997). Similar results were also reported in the pig-to-human *in vitro* model (Zhao et al., 1994).

1.3.9 Animal studies using a complement inhibitor

Understanding of the activation of complement leading to HAR encouraged *in vivo* animal studies. Direct inhibition of complement activity by administering anti-complement agents to the recipients has been attempted, including sCR1, CVF, FUT-157, K76, C3-binding peptide, and anti-C5 monoclonal Abs. The most promising agents are sCR1 and CVF.

sCR1 acts to inhibit human complement receptor 1 and has proven to be effective in preventing HAR in small animal studies. Based on immunosuppressive therapy with cyclosporine, cyclophosphamide and steroid, the addition of sCR1 prolonged the survival of porcine cardiac grafts to 11-32 days in monkey, in comparison with 38min in the control group (Davis et al., 1996). CVF is purified from the snake *Naja naja kaouthia* and acts as a complement activating protein to form stable C3/C5 convertase and exhaust the complement system. In the pig-to-primate cardiac xenograft model, CVF extended survival from less than 1 hour to medians of 5 days and 12 days, depending on whether splenectomy was performed. Dense deposition of IgM and, to a lesser extent, IgG and IgA, were seen on the ECs, but only trace levels of complement deposition were present in CVF-treated recipients (Kobayashi et al., 1997).

1.3.10 Genetic engineering for expression of a complement-regulatory protein

Despite the effectiveness of complement inhibitors, their effects are offset because of the cost, side-effects and potential compromise of the recipient's innate immune defenses. To avert the side-effects from systemic administration of a complement inhibitor, direct

surface targeting of hCRP by genetic modification was proposed. These include MCP (CD46), DAF (CD55) and protectin (CD59). As shown in Figure 1.3-1, CD59 limits formation of the terminal MAC. CD55 and CD46 may be the most useful because they not only inhibit the production of C3b but also limit the production of the proinflammatory peptides C3a, C4a and C5a. CD55 is generally considered to control the classical complement activation pathway more effectively and CD46 the alternative activation pathway (Christiansen et al., 1996; Loveland et al., 1993). However, CD46 is very effective at protecting cells against complement-mediated lysis by both the classical and alternative pathways, and its recognized ability to control alternative pathway activation suggested that for long-term graft survival, where innate immune responses might be important, it may be preferred over CD55 (Brodbeck et al., 2000).

The generation of pigs that express a hCRP, including CD46, CD55, and CD59 became possible and provided a major advance in xenotransplantation. CD59 transgenic pigs were first generated and high-level cell surface expression of CD59 was achieved in a variety of porcine cell types, most importantly on both large vessel and capillary ECs (Fodor et al., 1994). CD55 transgenic pigs were tested in cardiac xenograft models with immunosuppressants; the grafts escaped from HAR (McCurry et al., 1995). The grafts from CD46 transgenic pigs did not succumb to HAR, and survival extended for up to 23 days with the immunosuppressants of cyclosporine, steroids and cyclophosphamide (Diamond et al., 2001).

Another issue of debate concerned whether pig CRPs can regulate human complement as effectively as the human counterparts. The pig analogues of hCRPs are comparable to the human proteins in their capacity to inhibit human complement. The effectiveness of CRPs to protect xenografts depends on an increase in the number of CRPs expressed on the transgenic tissue (Morgan et al., 2005).

1.4 ACUTE HUMORAL XENOGRAFT REJECTION

Although several strategies have been developed to overcome the problems of HAR and prolong graft survival, the grafts are still lost within weeks or months due to another form of humoral rejection. This type of rejection is characterized by ECs activation, progressive infiltration of inflammatory cells, microvascular thrombosis, and ischemic changes.

AHXR, designated as delayed humoral xenograft rejection (DXR) or acute vascular rejection (AVR), usually occurs after 24 hours to within weeks after transplantation, causing the development of a consumptive coagulopathy and graft failure. Like its terminology, this phase of rejection is a heterogeneous phenomenon and the factors involved in this rejection remain undefined. The presumed mechanism for AHXR is Ab-mediated rejection with possible involvement of complement and /or infiltration of polymorphonuclear granulocytes. The factors contributing AHXR could be natural (Gal or non-Gal) or elicited XNA (Gollackner et al., 2004). AHXR is associated with the deposition of Abs and could be completely inhibited if the preformed Abs is depleted completely and elicited Abs production and complement activation are significantly

suppressed by using severely toxic immunosuppressants (Lin et al., 1998). On the other hand, the role of complement in AHXR remains controversial. In the pig-to-primate model using complement-regulator transgenic pigs, grafts undergoing complement-dependent rejection showed pathophysiologic mechanisms similar to HAR, with a prominent infiltration of inflammatory cells. In contrast, grafts succumbing to complement-independent AHXR contained almost no infiltration of lymphocytes, macrophages or NK cells. Therefore, a clear understanding of the role of complement in AHXR has significant therapeutic implications (Lin et al., 1998; Waterworth et al., 1998).

1.5 CELLULAR AND CHRONIC REJECTION

Acute cellular xenograft rejection occurs later after transplantation and appears to be similar to allograft rejection, but stronger. The mechanisms of cell-mediated rejection have less intensely been investigated because the strength and immediacy of AHXR has made it difficult to keep discordant recipients surviving long enough to study cell-mediated rejection until recently. Several cell types have emerged to be associated with cell-mediated xenografts rejection, such as CD4 T cells, NK cells, and other inflammatory cells (Auchincloss and Sachs, 1998).

Taken together, when transplanting pig organs into primates, activation of PAECs is initiated by the immunologic response and develops procoagulant characteristics compared with anticoagulant features in the quiescent condition. These activated PAECs play an essential role in a deleterious chain reaction during xenograft rejection and emerge as a major barrier for successful xenotransplantation.

1.6 ENDOTHELIAL CELL (EC) ACTIVATION

The EC surface in an adult human is composed of approximately $1 \text{ to } 6 \times 10^{13}$ cells, weighs approximately 1 kg, and covers a surface area of approximately 1 to 7 m² (Augustin et al., 1994). They have been found to operate several active functions rather than just provide physical separation between blood flow and tissue, which was thought in the earlier age. The ECs serve as a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic, and immunologic functions (Cines et al., 1998). The ECs exist in two states: quiescent and activated. When confronted by an inflammatory stimulus, ECs modify their phenotype and become an activated phenotype to promote adhesion, activation, and transmigration of circulating leucocytes, platelets and other cells (Cines et al., 1998; Pober and Cotran, 1990). This process is accomplished primarily via the up-regulation of a series of proinflammatory genes such as E-selectin, P-selectin, inter-cellular adhesion molecule 1 (ICAM-1, CD54), and vascular cellular adhesion molecule 1 (VCAM-1, CD106). The expression of these genes is regulated primarily at the transcriptional level through the activation of the transcription factor NF- κ B (Wrighton et al., 1996).

Of relevance to xenotransplantation, the donor ECs of grafts are the first site of contact between the recipients' blood and transplanted organ, and on which the inflammatory and immunologic response occur during HAR and AHXR. Therefore, they are divided into two phases, according to the timeframe of activation.

1.6.1 Type I activation

Type I activation of ECs, independent of protein synthesis, occurs within seconds to minutes, and has been postulated to be the underlying cause of HAR (Bach et al., 1995). Type I activation is typically mediated by ligands that bind to the extracellular domains of heterotrimeric G-protein-coupled receptors (GPCRs) and an elevated plateau of cytosolic Ca^{2+} levels, which usually last for 10–20 minutes, after which the receptors become desensitized, preventing restimulation (Pober and Sessa, 2007). Recipient XNA and complement result in the activation of porcine ECs, leading to retraction of endothelium and capillary leakage with consequent hemorrhage and exposure of subendothelial matrix. Collagen and vWF on the subendothelial matrix, combined with P-selectin and vWF on porcine ECs adhere to platelets via the receptor glycoprotein Ib (GPIb) on the platelets. In addition, activated platelets increase the expression of P-selectin and GPIIb/IIIa by inflammatory mediators to recruit platelets and promote thrombosis. The intensity and rapidity of HAR is catastrophic, resulting in the thrombosis of the graft, which limits further consumption of platelets and coagulation factors, therefore averting systemic coagulopathy.

1.6.2 Type II activation

Type II activation of ECs requires transcription and translation of new proteins; hence needs longer time to be initiated compared with type I activation (hours as opposed to minutes). This type of activation is responsive to the stimulation of the mediators such as tumor necrosis factor (TNF) and interleukin-1 (IL-1). In spite of being through different signaling pathways, they activate the same transcriptional factor, nuclear factor- κ B (NF-

κ B), and activator protein 1 (AP1)(Martin and Wesche, 2002). After activation, ECs synthesize adhesion proteins, cytokines, chemokines and procoagulant factors (Collins et al., 1995). Adhesion molecules such as selectins, ICAM and VCAM promote the adhesion and infiltration of leucocytes, which is much more effective following type II activation than type I activation. Initially, neutrophil recruitment is triggered by the synthesis and display of chemokines. The expression of adhesion molecules favors a transition from neutrophil-rich infiltrates to mononuclear cell-rich infiltrates, which typically occurs between 6 and 24 hours after cytokine-mediated activation (Munro et al., 1989). During AHXR, progressive accumulation of NK cells and macrophages, associated with the up-regulation of P-selectin and E-selectin is found in the grafts (Blakely et al., 1994).

Of importance, this type of activation causes ECs to become procoagulant, resulting in thrombosis. The *de novo* synthesis of TF initiates the coagulation cascade by binding to factor VIIa (FVIIa). Moreover, activated ECs lose thromboregulatory mechanisms such as the action of the ecto-NTPDases, thrombomodulin, heparan sulfate, contributing to the prothrombotic environments.

In xenotransplantation, AHXR was considered to be related with type II activation of ECs (Bach et al., 1995). The mechanisms that could result in type II activation still remain perplexing. Primate serum induces type II activation of PAECs (up-regulation of selectins or VCAM-1), dependent on the presence of complement (Solvik et al., 2001). This type of activation is associated with the binding of the IgG3 subclass of anti-Gal Abs (Saethre

et al., 2002) or anti-nonGal Abs (albeit less contributory) (Saethre et al., 2006). However, direct targeting of the Gal epitope by agonist could evoke type II EC activation in the absence of complement activation (Palmetshofer et al., 1998). Another study also showed induction of IL-8 and plasminogen activator inhibitor-1 (PAI-1) in PAECs after activation with XNA without the involvement of complement (Vanhove and Bach, 1993). On the other hand, whether type II activation of ECs is attributed to factors other than Abs or complement remains under investigation. It is speculated that platelets activated by retracted PAECs secrete chemokines to recruit and activate host monocytes and NK cells. The latter activated mononuclear cells secrete further cytokines, such as TNF- α , interleukin 1, and interferon- γ (IFN- γ). These then provide a stimulus for EC activation, with consequent coagulation and inflammation.

1.7 HAEMOSTASIS

Haemostasis is the process that maintains the integrity of a closed, high-pressure circulatory system after vascular damage. Vessel-wall injury and the extravasation of blood from the circulation rapidly initiate thrombosis in the vessel wall that seals the breach. Physiologically, this process requires circulating platelets and the coagulation system. Platelets are recruited to the site of injury, where they become a major component of thrombus and the coagulation cascade culminates in the generation of thrombin and fibrin (Furie and Furie, 2008).

During injury, platelets establish adhesive interactions with exposed subendothelial structures and are activated to become the reactive surface for continuing platelet deposition. Thrombus formation is initiated by platelet adhesion to extracellular matrix

and augmented by subsequent aggregation via the engagement of receptors of platelets (Ruggeri, 2000). Blood coagulation is initiated by TF to form thrombin and fibrin strands, which strengthen the platelet plug and serve as another potent stimulator to platelets via protease-activated receptor 1 (PAR1) in human and PAR4 in mice (Shrivastava et al., 2007). These occur concomitantly and are controlled by regulatory mechanisms, which are activated to terminate clot formation and commence fibrinolysis, temporally containing the thrombus formation. When pathologic processes disrupt the balance, excessive quantities of thrombin form and initiate thrombosis.

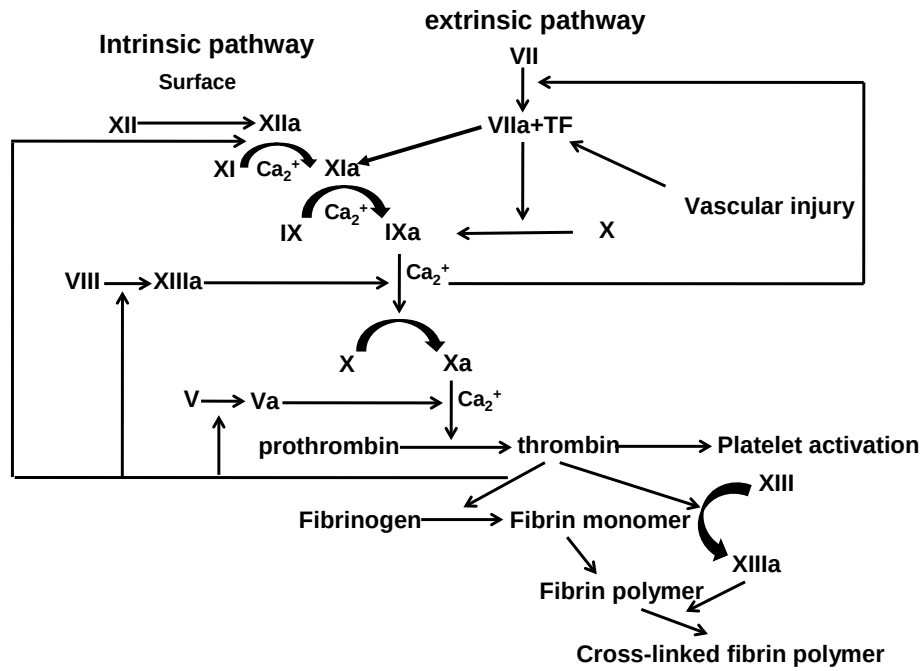
1.7.1 Coagulation pathways

The coagulation cascade consists of a series of proteolytic reactions, converting inactive pro-enzymes (zymogen) into active enzymes, resulting in the precise and balanced generation of thrombin, the main effective enzyme of the coagulation cascade. Most of them are serine proteases except factor V (FV) and factor VIII (FVIII), which are glycoproteins and Factor XIII (FXIII), a transglutaminase.

The coagulation cascade has two pathways: the contact activation pathway (formerly known as the intrinsic pathway) and the TF pathway (formerly known as the extrinsic pathway). The intrinsic pathway begins with the formation of the primary complex on collagen by high-molecular weight kininogen (HMWK), prekallikrein, and factor XII (FXII, Hageman factor). The extrinsic pathway is initiated by TF. Both converge to the common pathway at the activation of FX, which converts prothrombin to thrombin (Figure 1.7-1).

The extent of the haemostatic response is controlled by the action of inhibitory pathways, which ensure that thrombin activity and the spread of the haemostatic plug is limited to the site of vessel damage. The inhibitory pathway is operated by regulators such as TFPI, anti-thrombin or thrombomodulin.

A



B

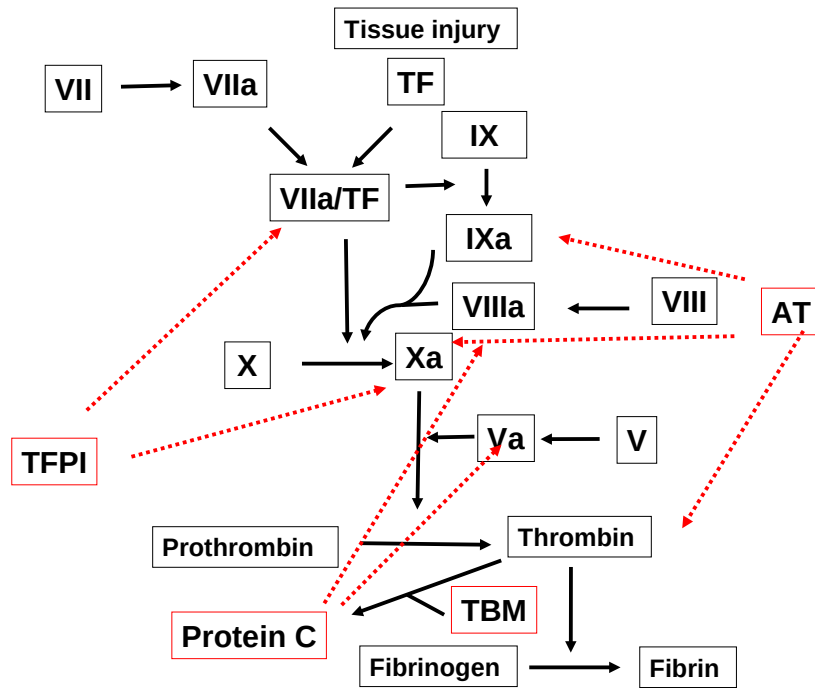


Figure 1.7-1 The cascade theory of coagulation pathway

A. Intrinsic and extrinsic pathways.

B. Regulators of coagulation pathway.

Red dashed line: inhibitor of coagulation. AT: antithrombin, TBM: thrombomodulin

TFPI: tissue factor pathway inhibitor. (Adapted from Mackman N, *Arterioscler Thromb Vasc Biol* 2007; Black line: stimulatory; red line: inhibitory)

1.7.2 Tissue factor (TF)

TF is a 47-kDa integral transmembrane protein and is considered to be the most important initiator of intravascular coagulation. When exposed to the blood stream, TF binds the coagulation factor VIIa (FVIIa), and the complex TF-FVIIa is a powerful activator of both FX and FIX, thereby initiating thrombin and fibrin formation (Mackman, 2004).

TF is constitutively expressed by certain cells within the vessel walls and cells surrounding blood vessels, such as vascular smooth muscle cells, pericytes, and adventitial fibroblasts (Mackman et al., 2007). Thrombosis may result from a pathologic response to vessel wall injury. Under physiological conditions, vascular cells that are in contact with blood do not express TF. However, pathologic conditions lead to induced TF expression by a variety of vascular cells and this expression plays an important role in thrombosis even without exposure of blood to the subendothelium.

For example, TF protein was observed on ECs in the aorta of septic baboons (Lupu et al., 2005). TF antigen has also been observed on circulating ECs in patients with sickle cell

disease and on ECs of the pulmonary vein in a mouse model of sickle cell disease (Solovey et al., 1998; Solovey et al., 2004). Furthermore, in a rodent septic model induced by systemic lipopolysaccharide, transgenic mice expressing TFPI or anticoagulant hirudin on endothelium were resistant to intravascular thrombosis and consumptive coagulopathy (Chen et al., 2004a). This observation suggests ECs are inducible to express TF activity by pathologic conditions.

TF expression has been shown to contribute to disseminated intravascular coagulation (DIC) in baboon and rodent models of sepsis. A key cell type that can be induced to express TF is the monocytes (Pawlinski et al., 2004; Semeraro and Colucci, 1997; Taylor et al., 1991). Although there have been some debates, several studies demonstrated robust TF expression on neutrophils in the different diseases (Higure et al., 1996; Maugeri et al., 2006).

Whether or not platelets express TF has been controversial. Platelets were demonstrated to confer TF activity through a process in which monocyte-derived microparticles bind to activated platelets, and activated platelets themselves release TF-positive microparticles (Falati et al., 2003; Rauch et al., 2000). However, other evidence shows that resting and activated platelets express TF activity and contribute to the pool of circulating TF (Losche, 2005; Siddiqui et al., 2002; Schwertz et al., 2006).

Microparticles are small membrane fragments released from activated or apoptotic vascular cells such as leucocytes, ECs, platelets and smooth muscle cells. They contribute

to the circulating TF activity because TF antigen levels have been detected in human platelet-free plasma (Misumi et al., 1998). Importantly, levels of TF-positive microparticles are elevated in patients with a variety of diseases, including cardiovascular disease, diabetes, cancer, sickle cell disease, and endotoxemia (Mackman et al., 2007).

Although negatively-charged phospholipids enhance the association of FVIIa with TF, they are not essential for complex formation of TF-FVIIa (Wildgoose et al., 1992). TF-FVIIa complex may enhance $V_{\max}$ of the reaction to catalyze the conversion of factor X to factor Xa, then subsequently shortening the clotting time. The explanations for this condition are that the exposure of phospholipids may change the structure of TF-FVIIa complex and enhance its function, or phospholipids induce a change in shape of TF-FVIIa complex can increase the number of active catalytic sites (Bach, 2006).

Under normal conditions, most membrane TF expose at the cell surface is encrypted. Recently, in addition to an initiator of blood thrombosis dubbed the procoagulant form of TF, TF was found to have another form (cryptic) because of disulfide isomerization (Ahamed et al., 2006). The surface-accessible, extracellular Cys¹⁸⁶–Cys²⁰⁹ disulfide bond of TF is critical for coagulation. When the disulfide bond of TF is cleaved by protein disulfide isomerase, TF also binds FVII to cleave and activate PAR2, the only receptor of PAR family that is not activated by thrombin and to exert as cell signaling. These signaling functions of TF-FVII in concert with thrombin elicit the cellular response to coagulation activation.

1.7.3 Thrombin

Following the initiation phase, the limited quantities of FXa that are generated catalyze the conversion of trace quantities of prothrombin to thrombin. Albeit in trace quantities, thrombin enables the feedback activation of FVIII and FV, non-enzymatic cofactors in the intrinsic pathway of the coagulation cascade. The complex activates further FX and, as a result, bypasses the reliance on TF·FVIIa as a source of FXa generation. Consequently, this allows the explosive generation of thrombin that enables this serine protease to fulfill its myriad functions, including the deposition of fibrin (Lane et al., 2005). Once generated, thrombin plays a central role in the regulation of thrombosis and termination of coagulation.

A primary function of thrombin is to catalyze the conversion of fibrinogen to fibrin. During this reaction, fibrin monomer is first generated and spontaneously polymerizes to form protofibrils which can undergo lateral aggregation. Together with activated platelet plugs, the local deposition of fibrin forms an extensive meshwork to form a stabilized clot that seals the site of vascular injury.

Thrombin is also a potent stimulator to activate platelets. During normal haemostasis, platelets are specifically recruited to the site of vascular injury by exposure of collagen and the actions of vWF. This forms the primary platelet plug. Thrombin specifically activates platelets by binding PAR1 receptor (Coughlin, 2005). Distinct to procoagulant function, thrombin also participates in the interaction of anti-coagulant function and inhibition of fibrinolysis, via thrombin-antithrombin complexes.

Of importance, evidence demonstrated that thrombin is involved in proinflammatory reactions and vascular neogenesis apart from coagulation. In peritonitis and the xenograft heart transplantation model, thrombin facilitates the generation of local monocyte chemoattractant protein (MCP)-1, which is needed to recruit natural killer cells via PAR1 receptor (Chen et al., 2008a; Chen et al., 2008b).

1.7.4 Coagulation inhibition

The activation of the coagulation cascade and platelets efficiently allows maintaining the integrity of the circulatory system. However, if unregulated, this cascade has the potential to generate overwhelming clot deposition and organ damage. Endogenous inhibitors ensure the balanced formation of thrombus, including TFPI, thrombomodulin/activated protein C and heparan sulfate.

In the initiation phase, TFPI is the principal inhibitor of TF-initiated coagulation, which binds and inhibits TF·FVIIa in a FXa-dependent manner (Bajaj et al., 2001). By targeting the initiating procoagulant stimulus, TFPI ensures that a small procoagulant stimulus does not induce the uncontrolled generation of thrombin. Since only a small quantity of FXa and thrombin is generated in this phase, this process is strongly influenced by the inhibitory activity of TFPI (Mann et al., 2003). During this propagation period, the thrombin facilitates fibrin deposition that stops extravasation of blood. The inhibitory actions of protein C and antithrombin pathways become more prevalent in controlling thrombin generation.

1.7.5 Tissue factor pathway inhibitor (TFPI)

TFPI is normally expressed by many cells; the major site is on ECs, which constitutively express the protein under normal conditions (Bajaj et al., 1990; Crawley and Lane, 2008). It has been reported that a proportion of TFPI is stored close to the membrane surface of cultured endothelial cells. This cellular fraction can be released in response to different agonists (eg, heparin, thrombin, shear forces). On secretion from endothelial cells, a small amount of TFPI may associate ionically with the cell surface proteoglycans via K3 and its basic carboxy terminus. Such an ionic interaction might explain the, albeit limited, competitive release of TFPI from the endothelial surface by heparin. The mobilization of intracellular/cell surface-associated TFPI into plasma contributes to the anticoagulant effect of heparin administration. Heparin not only induces TFPI secretion into plasma, it also upregulates TFPI gene expression leading to elevation of both cellular and plasma TFPI pools (Crawley and Lane, 2008).

The anticoagulant effect is mainly operated by TFPI α . The mature TFPI α is a 276 amino acid protein with three Kunitz-type domains (Wun et al., 1988). The first domain (K1) binds to the active site of FVIIa in the FVIIa/TF complex (Girard et al., 1989). The second (K2) binds and inhibits FXa (Petersen et al., 1996). The third Kunitz domain (K3) has no described inhibitory function, but may mediate the ionic binding of TFPI α to cell surfaces. The β isoform of TFPI-1, generated by alternative splicing, contains only K1 and K2 and is directly attached to ECs by a GPI linkage (Zhang et al., 2003).

TFPI secreted by endothelial cells circulates in plasma at a concentration of ≈ 2.5 nmol/L but fluctuates temporally in humans because of their natural circadian rhythms. Plasma levels are increased 1.5- to 3-fold after administration of heparin. The majority of the plasma pool circulates in association with low-density lipoproteins (LDL), and as a consequence confer markedly reduced inhibitory potential toward FXa and TF·FVIIa. LDL levels seem to correlate with plasma TFPI concentration. Only about 10% of plasma TFPI circulates in a full-length free 43-kDa form (Novotny et al., 1991).

1.7.6 Thrombomodulin and protein C activator

Thrombomodulin is expressed predominately on the vascular ECs and is widely distributed in the endothelium of arteries, veins, capillary and lymphatics (Maruyama and Majerus, 1987). It is a glycosylated transmembrane molecule of 557 amino acids and does not possess intrinsic enzymatic activity. When thrombin binds to thrombomodulin, the procoagulant activity of thrombin is altered. Protein C activation takes place by way of interaction of two receptors between the thrombomodulin–thrombin complex and the endothelial protein C receptor on endothelium (Figure 1.7-2). The binding of thrombin and thrombomodulin activates protein C much more efficiently (greater than 1,000-fold) (Dittman and Majerus, 1990). Activated protein C, together with cofactor protein S, provides an essential anticoagulant mechanism that prevents the amplification of thrombin generation via proteolysis of activated coagulation FVa and VIIIa.

As a result, it blocks the thrombin-mediated conversion of fibrinogen into fibrin and inhibits the binding of thrombin to other cellular receptors on platelets and inflammatory

cells. Recently, thrombomodulin was found to have important functions of fibrinolysis, inflammation and embryogenesis. For example, thrombomodulin is also a critical cofactor for the activation of thrombin-activatable fibrinolysis inhibitor, which inhibits plasmin formation (Weiler and Isermann, 2003).

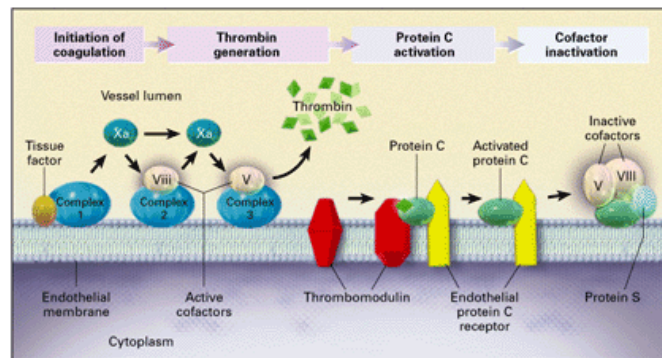


Figure 1.7-2 Endothelial activation of coagulation and protein C pathway

Coagulation is initiated by TF and other coagulation-factor complexes on the surface of endothelial cells. Thrombin is generated to form a complex with thrombomodulin. Protein C activation takes place by way of interaction between the thrombomodulin–thrombin complex and the endothelial protein C receptor (adapted from Faust SN, et.al. NEJM, 345: 408, 2001).

1.7.7 Heparan sulfate and antithrombin

Heparan sulfate achieves its anticoagulant activity by interacting with antithrombin, a serine proteinase inhibitor (serpin). After interaction, antithrombin is activated via a conformation change to inhibit blood coagulation. Antithrombin is a 58 kDa, 432 amino acid glycoprotein synthesized in the liver, with a half-life of 3 days (Johnson et al., 2006). Antithrombin inhibits all of the other proteolytic coagulation factors, but the

predominance of its anticoagulant activity is focused on the regulation of FXa, FIXa and thrombin.

Heparan sulfate is found on the surface of ECs and subendothelium. *In vivo*, form of heparan sulfate and antithrombin maintain a normal, non-thrombogenic nature (Quinsey et al., 2004). Antithrombin is expressed as both an α -form and a β -form. The α -form represents 90% of antithrombin. While comprising only 10% of antithrombin, β -form antithrombin has a higher affinity for heparin and is thought to exert an overall larger anticoagulant effect (McCoy et al., 2003).

1.8 MOLECULAR INCOMPATIBILITIS BETWEEN SWINES AND PRIMATES

Because of evolutionary divergence between primate and pig, there are some physiological barriers that remain to be overcome for successful xenotransplantation. Dysregulation of coagulation which is believed to contribute to consumptive coagulopathy after xenotransplantation is a major challenge (Cowan et al., 2000). The coagulation cascade is tightly controlled by coagulation regulators, which are mainly TFPI, protein C system/ thrombomodulin and antithrombin.

In the initiation stage of coagulation, activated FVII binds to TF and initiates a trickle of thrombin, which is regulated by the function of TFPI. However, *in vitro* porcine TFPI inefficiently inhibits human TF-dependent FX production and during AHXR, loss of porcine TF promotes the development of a procoagulant environment (Kopp et al., 1997).

This concept has been challenged by a recent study which showed porcine and human TFPI shared about 80% protein and over 80% in the functional Kunitz-1 and Kunitz-2 domains. The ability to inhibit human FVIIa/TF complex was also similar between both TFPIs (Lee et al., 2008). In the propagation stage, the coagulation cascade is mainly regulated by thrombomodulin/protein C. Although porcine thrombomodulin shares 69% sequence identity with human thrombomodulin and binds to human thrombin, it is a poor cofactor to activate protein C and thrombin-activatable fibrinolysis inhibitor, contributing to insufficient cleavage of FV and FVIII (Kopp et al., 1998a; Roussel et al., 2008).

In terms of platelet activation, porcine vWF is known to spontaneously aggregate human platelet, in the absence of shear stress via GPIb because of difference in the O-linked glycosylation pattern between porcine and human vWF A1 domain (Pareti et al., 1992) (Schulte Am Esch et al., 2005). On the other hand, porcine fgl-2 (fibrinogen-like 2), which is expressed on activated ECs, converts human prothrombin to thrombin without the involvement of the coagulation cascade (Ghanekar et al., 2004). In the rodent model, fgl-2(-/-) hearts protected grafts from the injury of AHXR (Mendicino et al., 2005). Ecto-NTPDases plays a pivotal role to maintain the balance of ADP and adenosine. During AHXR, the activity of CD73 is significantly reduced in pig heart and kidney tissue, which may have reduced capacity to generate adenosine and thus increased sensitivity to platelet aggregation (Cowan and d'Apice, 2008). Transgenic expression of CD39 on the surface of mouse ECs was developed to prolong graft survival without overt bleeding tendency in the rodent model (Dwyer et al., 2004).

Human cytokines produced by the infiltrating mononuclear cells in AHXR trigger the interaction between proinflammatory cells. One unpublished data described that human IFN- γ do not stimulate PAECs and such incompatibilities might attenuate immune response of xenografts (Bach et al., 1996). Nevertheless, mouse renal allografts lacking IFN- γ receptors rapidly developed ischemic necrosis because of massive damage to the microcirculation of the graft by infiltrating cytotoxic T lymphocytes (Sis et al., 2007). These observations support the hypothesis that INF- γ induced major histocompatibility complex class I proteins on donor tissue engage inhibitory receptors on recipient cytotoxic T lymphocytes and prevent them from injuring the microcirculation by perforin–granzyme mechanisms. Therefore, incompatibility of cytokines may indirectly promote coagulopathy in xenotransplantation.

1.9 INTERACTIONS BETWEEN INFLAMMATION AND COAGULATION

Inflammation and coagulation play pivotal roles and are intertwined in the pathogenesis of vascular disease. Inflammation results in activation of coagulation; but coagulation also considerably affects inflammatory activity. For example, the development of systemic coagulation activation, which results from the systemic inflammatory response to severe infection or sepsis contributes to multiple organ dysfunction. Conversely, coagulation factors such as thrombin, FVII and protein can also substantially modulate inflammatory activity.

1.9.1 TF expression on inflammatory cells

The thrombin generation during inflammation is TF-dependent. Blocking TF activity completely abrogates coagulation activation in models of experimental endotoxemia or bacteremia, whereas anti-FXII monoclonal Ab that inhibits intrinsic pathway has no effect on thrombin formation (Levi et al., 1994; Pixley et al., 1993). The sources of TF may be different in various diseases and in various inflammatory situations. In atherosclerotic plaques, macrophages produce abundant TF at their surface and release it into blood upon plaque rupture. The pathophysiology of DIC was demonstrated to be mediated by the expression of TF. Many diseases such as cancer, severe trauma, and obstetric disorders are associated with DIC, and TF expression has been detected in the involved cells (Levi and Ten Cate, 1999).

1.9.2 Downregulation of anticoagulation system during inflammation

During inflammation, anticoagulation properties of TFPI, thrombomodulin and antithrombin can be impaired. The level of antithrombin dramatically reduces because of impaired synthesis and rapidly consumption after initiation and ongoing formation of thrombin (Levi et al., 2004). Proinflammatory cytokines also cause reduced synthesis of heparan sulfate (glycosaminoglycans) on the EC surface, which will also contribute to reduced antithrombin function (Kobayashi et al., 1990). Moreover, the protein C system is suppressed, mainly owing to downregulation of thrombomodulin at the EC surface, mediated by the proinflammatory cytokines TNF- α and IL-1 β (Nawroth and Stern, 1986). The expression of TFPI was also shown to be inhibited by cytokines in rodent model (Shimokawa et al., 2000).

1.9.3 Inflammation inhibits fibrinolysis

On the other hand, the fibrinolytic response is exerted by the release of plasminogen activators from storage sites in vascular ECs upon the formation of fibrin. However, PAI-1 is increased and subsequent plasmin generation is counteracted (van der Poll et al., 1991). As a result, during inflammation fibrinolysis is inhibited and inadequate fibrin is removed, thereby contributing to microvascular thrombosis.

1.9.4 Interactions between coagulation and inflammation

In addition to TF and thrombin, fibrinogen and fibrin can directly stimulate expression of proinflammatory cytokines (such as TNF- α and IL-1 β) on mononuclear cells and induce production of chemokines (including IL-8 and MCP-1) by ECs and fibroblasts (Szaba and Smiley, 2002). Once thrombin is formed, this will activate additional platelets. Activated platelets mediate the adherence of platelets to leucocytes and ECs by the expression of P-selectin on the surface. Subsequently, the binding of activated platelets to neutrophils and mononuclear cells will enhance the expression of TF on monocytes via the pathway of NF- κ B activity.

The intricate interactions between inflammation and coagulation may affect each other. This cross-talk occurs at the levels of thrombin formation, platelet activation, leucocytes activation and cytokine interaction, resulting in inflammation to become critical conditions such as DIC. Increased insight into the molecular mechanisms in the close

relationship between inflammation and coagulation may contribute to the identification of new targets for therapies that can modify excessive activation in this system.

1.10 PLATELETS

Platelets are the principal effectors of cellular homeostasis in human and other mammals. During injury, they adhere to exposed subendothelial matrix (fibrinogen) and aggregate in response to prothrombotic signaling. As a result, the formation of platelet-fibrin clots seals vascular disruptions, which is critical for ultimate wound and vessel repair.

In the course of evolution, ancient homeostatic functions were operated by a cell or a small number of cell types rather than a specialized cellular repertoire. In many invertebrates, a single type of cell performs innate defensive functions and aggregates to have primitive wound-sealing functions. By the vertebrate stage, distinct specialized types of cells were developed in blood. In humans, platelets are anucleate cells that are derived from megakaryocytes in the bone marrow and then released into the circulation, where they remain for an average of 10 days. Eventually, they are either consumed in thrombus formation or destroyed in the spleen.

In addition to thrombosis, it has become evident that platelets also have relevant functions in inflammation by interplay with the immune system, which have been studied in vasculitis and atherosclerosis (May et al., 2008). Similar mechanisms may contribute to acute and chronic vascular lesions in transplants. Thus, platelets play a pivotal role both in thrombosis and inflammation.

1.10.1 Platelets and primary haemostasis

At the site of injury, platelet adhesion occurs to form a monolayer plug of activated platelets through the receptor GPIb to newly exposed collagen fibrils within the vessel wall and soluble vWF. Further platelet activation results from other physiological stimuli, which are generated in the proximity of vascular injury. They include the formation of thrombin on the surface of activated platelets and a variety of substances from the granules of activated platelets. The substances include 1) ADP, adrenaline and serotonin to activate adherent platelets in an autocrine manner by amplifying integrin adhesiveness, 2) fibronectin and thrombospondin that reinforce and stabilize the platelet aggregation, 3) fibrinogen to serve as a further source at the site of vascular damage for reinforcing platelet clot, and 4) thromboxane A₂ to allow vascular constriction and further platelet activation and aggregation. Activated platelets undergo a shape change and stick to each other via bridges formed by the GPIIb/IIIa integrin and multivalent adhesive proteins such as fibrinogen, fibrin, and vWF. This results in the accumulation of platelets into plugs and accelerates thrombus formation (Brass et al., 2005). Finally, platelets provide the exposure of procoagulant phospholipids to augment clotting reactions during the coagulation cascade (Bever et al., 1985).

1.10.2 von Willebrand factor (vWF)

vWF is a multifunctional adhesive glycoprotein stored in platelet α -granules and Weibel-Palade bodies of ECs. In its mature form, it consists of disulfide-linked, approximately 275-kDa subunits forming multimers of at least 20,000 kDa. Each subunit is composed of

conserved domains: D-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2. The A1 domain encompassing the disulfide bond at C1272–C1458 (C509–C695 in the mature sequence) contains the binding site for GPIb α , heparin, and other binding partners (Andrews et al., 1997). The vWF A3 domain binds collagen, while C1 contains an Arg-Gly-Asp (RGD) sequence that binds the receptor GPIIb/IIIa on platelets. The GPIb-binding site on vWF A1 is cryptic under static conditions, but becomes competent to bind receptor when exposed to shear force, or activated by non-physiological modulators. The interaction between vWF and GPIb is easily dissociated and not irreversible; subsequent activation of GPIIb/IIIa platelets eventually provides irreversible adhesion by binding to the RGDS sequence in the vWF C1 domain. Ultra-large vWF multimers expressed on activated ECs are highly prothrombotic and bind platelets with high avidity. Under shear stress, the metalloproteinase (a disintegrin and metalloproteinase with a thrombospondin type 1 motif-13, ADAMTS-13) cleaves within the A2 domain, between Y1605 and M1606, decreasing vWF size and downregulating GPIb-dependent platelet adhesion (Andrews and Berndt, 2008).

1.10.3 Interactions between platelets and endothelium

Like leucocytes, unactivated platelets were found to roll on activated endothelium in mesenteric venules. By intravital microscopy, the rolling process was first revealed on leucocyte movement, which is the initial step of leucocyte extravasation to the site of inflammation (Springer, 1994). Similarly, platelets rolling on endothelium dependent on shear rate are supported by P-selectin or vWF (Wagner and Frenette, 2008). One member of adhesion molecules, P-selectin is stored in granules of platelets (Berman et al., 1986)

and in Weibel-Palade bodies of ECs (McEver et al., 1989); then secreted and expressed on the cell surface of both platelets and ECs after stimulation. When shear rate is higher, e.g. in small venules or arterioles, the rolling process is supported by P-selectin (Frenette et al., 1995). In veins with lower shear rate, the process of adhesion was demonstrated to be mediated by vWF released from Weibel-Palade bodies on the endothelium and receptor of GPIb α on the platelets. The adhesion of murine platelets was exclusively inhibited by human GPIb α antagonist (Andre et al., 2000). On the other hand, the receptor of vWF, GPIb α , has also been shown to be a counter-receptor of P-selectin (Romo et al., 1999). This interaction of P-selectin is distinctive from that with P-selectin glycoprotein ligand 1 (PSGL-1), requiring neither calcium nor carbohydrate core-2 branching or alpha 1,3-fucosylation. This may thus suggest platelets roll on endothelium via P-selectin and GPIb α interaction (Polgar et al., 2005).

Whether E-selectin plays a role in the interaction between platelets and ECs is still unknown. The same group (Wagner DD) also demonstrated that E-selectin, whose expression is induced by cytokines, contributes to resting platelets rolling on endothelium and a ligand(s) for E- and P-selectins is constitutively expressed on the surface of platelets (Frenette et al., 1998). The ligand on the platelets to E selectin of ECs remains unidentified. Such a ligand is likely to bear specific carbohydrate moieties carried by a protein or a glycolipid scaffold. Interestingly, purified glycolipids (gangliosides) from human platelets contain sialyl-Lewis^x epitopes (Cooling et al., 1995), and similar glycolipids isolated from human myeloid cells have been shown to bind E-selectin (Stroud et al., 1996a; Stroud et al., 1996b).

1.10.4 Proinflammatory effects of activated platelets

Platelets contain various compartments, such as 3 different granules (α -granules, lysosomes, dense core granules), and a complex membranous system that allows them to store and rapidly release a variety of factors after stimulation. The molecules and functions are summarized in Table 1.10-1. These proteins act in a concerted and finely-regulated manner, influencing widely differing biologic functions, such as cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation, and proteolysis, all of which accelerate inflammatory processes and cell recruitment. Activated platelets enhance not only lymphocyte and NK cell adhesion and cells infiltration but also Ab production by B cells and the cytotoxicity of cytolytic T cells and NK cells (Li, 2008). The cross-talk is based on the interaction of P-selectin on platelets and PSGL-1 on leucocytes. Leucocytes are recruited into growing thrombi and promote fibrin deposition that is inhibited by Abs to P-selectin (Palabrica et al., 1992). Clotting was accelerated in P-selectin-knock-in mice that lack the cytoplasmic domain (Δ CT mouse) or the mice with high levels of soluble P-selectin in the plasma. These observations indicate that P-selectin plays an important role in a procoagulant state of the animals (Hartwell et al., 1998). Activated platelets were also demonstrated to induce monocytes and ECs to express TF activity. On the other hand, platelets also modulate humoral immunity by stimulating B cell proliferation and facilitating Ab production. Platelet-associated CD40L (CD154) was competent to induce the CD40-dependent proliferation of B lymphocytes in response to the GPIIb/IIIa when platelets and peripheral blood B lymphocytes from idiopathic

thrombocytopenic purpura patients with circulating anti-GPIIb/IIIa Abs were cultured together (Solanilla et al., 2005).

Location	Category	Molecule	Functions
α - granule	Adhesion	P-selectin	Adhesion platelets, ECs & leucocytes
		vWF	Facilitates rolling
	Chemokine	CCL3	Monocyte & T-cell recruitment
		CCL5	Monocyte, eosinophil & T-cell recruitment
		CCL7	Monocyte, eosinophil & T-cell recruitment
		CXCL4	Neutrophil & T-cell recruitment
		CXCL7	Neutrophil recruitment
		CXCL8	Neutrophil recruitment
		CXCL12	Lymphocyte & progenitor cell recruitment
	Cytokine	IL-1 β	Activates ECs & lymphocytes
		TNF- α	Activates ECs & increases vascular leakage
		TGF- β	Cell proliferation, differentiation, apoptosis, fibrosis
		CD40/CD40L	Maturation of antibody
	Growth factor	PDGF	Angiogenesis & fibrosis
Dense granule	Nucleotide	ATP/ADP	Stimulate platelets & endothelial cells
	Transmitter	Serotonin	Stimulates platelets & T cells
Lysosomes	Coagulation factors	Factor V	Coagulation
		Factor XI	Coagulation
		Protein S	Anti-coagulation
		Plasminogen	Fibrinolysis
		PAI-1	Antifibrinolysis
Membrane	Ligand receptors	PSGL-1	Ligand for P-selectin
		PAR 1	Receptor for thrombin
		ADP receptor	Stimulates platelets
		GPIa	Binds to collagen
		GPIb	Binds to collagen
		GPIIb/IIIa	Binds to vWF & fibrinogen
		GPIV	Binds collagen strongly

Table 1.10-1 Partial list of granular contents and membrane expressed receptors on human platelets that are relevant to organ transplantation. (Adapted from Morrell et al., AJT 2007.)

1.10.5 Platelets synthesize *do novo* proteins through pre mRNA splicing

Gene expression is regulated at several checkpoints. A critical step is to remove noncoding introns from newly-transcribed pre-mRNAs. This process occurs cotranscriptionally in the nucleus by a *trans*-acting complex termed the spliceosome (Maniatis and Reed, 2002). The human spliceosome contains small nuclear ribonucleoproteins (snRNPs) and auxiliary proteins that impart the correct architecture necessary for proper intron recognition and excision. Splicing ensures that mature, translatable mRNAs are available for transportation to cytoplasm. Initially, the study indicated that this process was operated within the nucleus. Denis et al. proved that the process of splicing of pre-mRNAs could be operated beyond the boundary of the nucleus and occur in anucleate cells, such as platelets, which also confer the spliceosome component (Denis et al., 2005). In this way, platelets were found to synthesize the *de novo* proteins, such as Bcl3, interleukin-1, PAI-1, and TF after activation (Brogren et al., 2004; Lindemann et al., 2001; Panes et al., 2007; Schwertz et al., 2006; Weyrich et al., 1998).

1.10.6 Receptor of glycoprotein IIbIIIa (CD41)

The platelet integrin (GPIIbIIIa) is the final common pathway of platelet activation, which not only binds fibrinogen and vWF to mediate platelet aggregation and adhesion, but also as a signaling receptor. Platelet agonists, such as ADP, thrombin and collagen, induce “inside-out” signaling which activates the receptor function of GPIIbIIIa for soluble fibrinogen. Subsequent platelet aggregation leads to “outside-in” signaling,

inducing platelet aggregate stabilization and triggering a variety of functions important to platelet physiology.

During the process of outside-in signaling, thromboxane A₂ is generated and functions in an autocrine loop to stimulate platelets and confer the ability of vasoconstriction. The molecules in the α or dense granules are secreted and released to appear in autocrine loops that augment further platelet aggregation and adhesion (Phillips et al., 1991).

1.10.7 Adenosine diphosphate (ADP) receptor

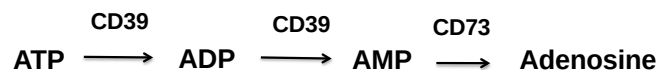
ADP is an important physiological molecule that plays a vital role in normal haemostasis and thrombosis. Despite being a weak agonist and only inducing reversible platelet aggregation compared to thrombin or collagen, ADP is an important so-called secondary agonist which amplifies most of the platelet responses and contributes to the stabilization of thrombus because of its presence in large amounts of the dense granule of platelets and injured vascular sites (Gachet, 2008). Platelets possess three P₂ receptors for adenine nucleotides: P₂Y₁ and P₂Y₁₂, which interact with ADP, and P₂X₁, which interacts with ATP. ADP-induced platelet activation is initiated via the platelet P₂Y₁ receptor, which is coupled to G α_q and phospholipase C and mediates calcium mobilization from internal stores, shape change and an initial aggregation response. Platelet activation and the aggregation response are amplified and sustained by the P₂Y₁₂ receptor, which is coupled to G α_i , leading to inhibition of adenylate cyclase and activation of phosphoinositide-3-kinase, Rap1b, Akt and potassium channels (Jackson et al., 2005). P₂X₁ receptor is an ion channel that causes a rapid influx of calcium and platelet shape change in response to

ATP and contributes to the platelet activation induced by low concentrations of collagen (Burnstock, 2007).

The P2Y1 and P2Y12 receptors are also differently involved in the procoagulant activity of platelets (Storey et al., 2000). Whilst both receptors are indirectly associated with the role in platelet P-selectin exposure and in the formation of platelet-leucocyte aggregates leading to leucocyte tissue factor exposure, the P2Y12 receptor is also directly implicated in the exposure of phosphatidylserine at the surface of platelets (Leon et al., 2003). Moreover, administration of P2Y12 antagonists limits the ability of platelets to adhere to and activate leucocytes and reduces the release of important mediators such as sCD40L (Judge et al., 2005).

1.10.8 ECs generate adenosine

In addition their presence in dense granules of platelets, nucleotides are present in intracellular fluid as a consequence of cell lysis, selective permeabilization of the plasma membrane and exocytosis of secretory vesicles, such as dense bodies. Ecto-NTPDases are a family of ectonucleotidases, which largely expressed by ECs in the vascular environment, sequentially converts extracellular purine nucleotides in the blood, such as ATP and ADP, to the monophosphate form (AMP).



CD39 is the main E-NTPDase in the endothelium of the vasculature system. The ectonucleotidase cascade is initiated by CD39, hydrolyzing ATP and ADP to AMP. Then

AMP is converted to adenosine via CD73. Both of them closely regulate the local and extracellular plasma concentration of adenosine (Goding et al., 1998). Through regulating ADP metabolism, ecto-NTPDases become one of the important pathways by which ECs influence the behaviour of platelets. ADP is a platelet agonist that triggers aggregation, augments the action of other platelet agonists, and mediates adrenaline release to induce vasoconstriction, while adenosine has opposing effects and creates an innate protective environment. Thus, by actively suppressing platelet-EC interactions and promoting downstream generation of adenosine, CD39 acts in conjunction with locally released mediators, such as prostacyclin and nitric oxide (NO), to maintain the balance between proinflammatory and anti-inflammatory factors.

1.11 ANTI-PLATELET AGENTS

The regulation of platelet function, and thereby haemostasis and thrombosis, is a precarious balance between activation and inhibitory mechanisms that control platelet activation upon exposure to damaged tissues, yet enable platelets to remain quiescent in the undamaged circulation. Understanding of these mechanisms (agonists, receptors and signaling pathways) is essential for the development of more effective anti-platelet therapies. Several drugs have been developed to prevent or treat vascular thrombotic diseases through different mechanisms (Figure 1.11-1).

1.11.1 Cyclooxygenase inhibitors

The product of cyclooxygenase, prostacyclin, is a vasodilator prostanoid that inhibits platelet activation in response to a variety of agonists. Conversely, thromboxane A₂ is a

potent vasoconstrictor and activator of platelets. In a murine model of catheter-induced carotid injury, vascular proliferation and platelet activation were enhanced in prostacyclin receptor (-/-) mice but were inhibited in thromboxane A₂ receptor (-/-) mice (Cheng et al., 2002). Low-dose aspirin reduces production of thromboxane A₂ from platelets by 97 to 99% in humans, (Davi and Patrono, 2007) and this reduction has been similar to the effect in a mouse model with hypomorphic COX-1 alleles (COX-1^{low} "knockdown"). The 97% decline in production of platelet thromboxane A₂ in these mice decreased platelet aggregation and prevented arterial thrombosis induced by photochemical injury (Yu et al., 2005).

1.11.2 ADP receptor blocker

The interaction of ADP with its platelet P2Y₁₂ receptor is essential for platelet activation. P2Y₁₂^{-/-} mice have a prolonged bleeding time and are protected against arterial thrombosis induced by ADP or a PAR-4-activating peptide (Andre et al., 2003). P2Y₁₂ blockers, such as ticlopidine and clopidogrel, were invented to interfere with this interaction. The effects on reducing the risk of major vascular events were similar to that achievable with low-dose aspirin (Patrono et al., 2004).

1.11.3 Blocker of CD41 receptor

Since GPIIb/IIIa plays a pivotal role in the final common pathway leading to platelet aggregation, inhibitors or Abs of this integrin have been expected to prevent platelets aggregation. Many studies showed GPIIb/IIIa inhibitors to reduce morbidity and mortality in patients with acute coronary syndromes (1998). Eptifibatide, a synthetic

1.12 STATIN

The statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, significantly reduce synthesis of cholesterol (Downs et al., 1998; Sacks et al., 1996). Clinical trial evidence demonstrates statins reduce the progression of atherosclerosis and may lead to the regression of plaque and reduction in stenosis. This benefit in coronary cardiovascular disease was thought to be associated with the lowering of cholesterol. However, recent studies indicated the clinical benefit of statin is disproportionate to the rather modest improvement seen in luminal diameter. These observations suggest statins have other lipid-independent effects, such as immuno-regulatory, anti-inflammatory, and anticoagulant actions (LaRosa, 2001; Palinski, 2001).

1.12.1 Pharmacology

HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway, through which cells synthesize cholesterol from acetate moieties (Figure 1.12-1). Inhibition of HMG-CoA reductase, and the subsequent reduction in cholesterol synthesis, results in hepatocytes increasing their surface expression of LDL-receptors, so increasing uptake of LDL and reducing plasma LDL and cholesterol. The statins exert their action because of their ability to bind to HMG-CoA reductase more potently than HMG-CoA (Istvan ES, 2001). Apart from the reduction in cholesterol biosynthesis, interference with the mevalonate pathway also prevents the synthesis of isoprenoid intermediates, including FPP (farnesylpyrophosphate) and GGPP (geranylgeranylpyrophosphate) (Goldstein and Brown, 1990). Geranylgeranyl and farnesyl are lipid attachments important in the post-translational modification of signaling proteins, including the small GTPases Rho, Rac

and Ras and the G-proteins (Casey, 1995). These molecules may alter cell signaling, membrane stability, membrane trafficking and gene transcription.

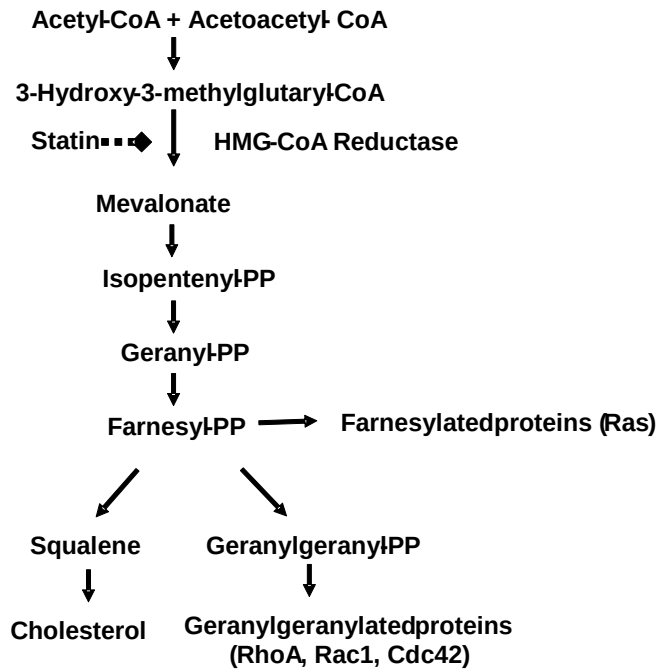


Figure 1.12-1 The pathway of cholesterol synthesis

Inhibition of HMG-CoA reductase by statins has multiple downstream effects, including inhibition of cholesterol biosynthesis and of isoprenoid intermediates such as FPP and GGPP. Geranylgeranyl and farnesyl are lipid attachments important in the post-translational modification of signalling proteins, including the small GTPases Rho, Rac and Ras and G proteins. PP, pyrophosphate.

1.12.2 Statin function on ECs

The beneficial effects of statins on vascular endothelium have been reported within 1 month of starting treatment and in the absence of lipid lowering. The lipid –independent

benefit is associated with NO bioavailability. Administration of statins augmented stimulated and basal NO dilator functions of the endothelium (O'Driscoll et al., 1997). Endothelial NO is a vasodilator, inhibits smooth muscle proliferation, platelet aggregation, endothelial adhesion molecule expression and leucocyte–EC interactions. The demonstration that statins are able to enhance local NO generation in ECs, by increasing the half-life of eNOS (endothelial NO synthase) mRNA, contributed to the acceptance for lipid-independent effects of statins (Landmesser et al., 2004). In addition, the evidence that statins inhibited the expression of the potent vasoconstrictor endothelin-1 in ECs through Rho proteins supported the beneficial effect of statins independent of lowering lipid (Hernandez-Perera et al., 2000).

1.12.3 Thrombotic and fibrinolytic effects

Statins have various effects on haemostasis and thrombosis, which may be especially important at sites of atherosclerotic plaque rupture (Rosenson and Tangney, 1998). The effect is attributed to inhibition of platelet activation, and maintenance of a favourable balance between prothrombotic and fibrinolytic mechanisms. Lipophilic statins (fluvastatin, simvastatin) suppress TF antigen and activity with the diminution of TF mRNA by cultured human macrophages through inhibition of a geranylgeranylated protein (Colli et al., 1997). The evidence was also confirmed in an animal study that showed that statins alter plaque biology by reducing proliferation and expression of MMP and TF of macrophages (Aikawa et al., 2001). Similarly, simvastatin prevents TF induction of human aortic ECs and vascular smooth muscle cells (VSMCs) by thrombin, at least in part through inhibition of Rho-kinase-dependent Akt dephosphorylation (Eto et

al., 2002). The inhibition of TF by statins has also been demonstrated in an animal study (Baetta et al., 2002). Another anti-thrombotic effect is that statins encourage fibrinolysis. Lovastatin, fluvastatin, atorvastatin and simvastatin have been shown to increase the profibrinolytic factor, tissue plasminogen activator (tPA), while inhibiting synthesis of the antifibrinolytic PAI-1 by ECs, macrophages and VSMCs (Bourcier and Libby, 2000; Lopez et al., 2000).

1.12.4 Inhibition of platelet aggregation

An important additional anti-thrombotic action is inhibition of platelet activation and function. Statins may reduce platelet aggregation by changing the cholesterol content of platelet membranes, which alters membrane fluidity by modulating cytosolic Ca^{2+} in platelets by affecting Ca^{2+} transport pathways (Le Quan Sang et al., 1995). In addition, a study has reported that lovastatin increases ecto-NAPDases activity and membrane expression in ECs, thus enhancing the inhibition of platelet aggregation at the EC surface through the action of the ecto-NAPDases product adenosine. This action appears to be independent of changes in membrane cholesterol (Hale et al., 1998; Laufs et al., 2000).

1.12.5 Immunoregulatory effects

Statins also exert immunoregulatory effects through lipid-lowering-independent mechanism. The function of macrophages and monocytes (Pahan et al., 1997; Romano et al., 2000), the activation and proliferation of B lymphocytes (Rudich et al., 1998) and cell cytotoxicity of T lymphocyte may be mitigated through inhibition of cytokine production and induction of NO synthase as shown *in vitro* and *in vivo* demonstrations

(Mason, 2003). Furthermore, pravastatin, lovastatin and atorvastatin have been shown to inhibit IFN γ -induced expression of MHC class II in macrophages, ECs and VSMCs. This effect provides a scientific rationale for using statins as immunosuppressants in organ transplantation (Kwak et al., 2000).

In a study based on cardiac transplantation, pravastatin had beneficial effects on cholesterol levels, the incidence of rejection causing hemodynamic compromise, one-year survival, and the incidence of coronary vasculopathy (Kobashigawa et al., 1995). In a pilot study, statin also reduced episodes of biopsy proven acute rejection in kidney transplant recipients treated with cyclosporine-based immunosuppression (Katznelson et al., 1996).

Although these effects require larger scale studies, the pleiotropic functions of statins offer the opportunities to link between different areas of medicine. It may turn out to be of great interest to explore the role of statins in xenotransplantation, where multiple challenges have to be dealt with simultaneously.

1.13 SUMMARY

Complement activation and coagulation has been recognized as pivotal issues for successful xenotransplantation. The generation of the GT-KO pigs was a huge step forward, eliminating the target of a whole class of pre-existing complement-fixing antibodies and the source of a major contributor to the induced humoral response. However, in this chapter I have attempted to

show that the remaining challenges from non-humoral immune response such as molecular incompatibilities and unregulated coagulation are by no means insignificant , and provided evidence for potential logistic avenues to deal with major barriers for xenotransplantation.

CHAPTER 2

MATERIALS AND METHODS

2.1 ENDOTHELIAL CELLS

2.1.1 Harvest and cultures of porcine aortic endothelial cells (PAECs)

Thoracic aortas from wild-type (WT) or transgenic pigs were harvested immediately after euthanasia and stored in UW solution or RPMI-1640 supplement with 20% heat-inactivated (HI) fetal calf serum (FCS, Sigma, St. Louis, MO), penicillin (100 U/ml, Sigma) and streptomycin (25µg/ml, Sigma) at 4°C.

PAECs were obtained from freshly harvested porcine aortas by treatment with 0.05% collagenase B (Roche Applied Science, Indianapolis, IN). The cells were collected and washed with washing medium (RPMI-1640 containing 10% HI fetal bovine serum (FBS, Invitrogen, Carlsbad, CA)) to inactivate the collagenase, and were resuspended in PAEC culture medium (medium 199, Invitrogen) containing 10% HI FCS and antibiotic-antimycotic (Invitrogen) and endothelial growth factor (30µg/mL, BD Biosciences, San Jose, CA) and plated into collagen I-coated 6-well, 25 cm² or 75 cm² tissue culture flask (BD) at 37°C in 5% CO₂ (Appendix 1,2,3,4). Trypsin-EDTA (0.05% trypsin-0.02% EDTA, Invitrogen) was used to passage cells and all cells in the experiments were within passage 6.

2.1.2 Human ECs and breast cancer cell line

Human aortic ECs (HAECs) (as an allograft control compared to PAECs), purchased from Cambrex (Walkersville, MD) and a human breast cancer cell line (ZR-75-1; as a

positive allograft control for the expression of human TF) were cultured in EC basal medium (EBM-2, Lonza, Walkersville, MD) under the same conditions as PAECs

2.1.3 Phenotype of PAECs

The phenotype of PAECs was determined by fluorescence-activated cell sorter analysis using anti-CD31 Ab. PAECs (purity >90%) were used in the following experiments. The phenotypes of PAECs from WT, GT-KO or MCP (CD46, (+/+)) pigs were confirmed by flowcytometry (Figure 2.1-1). Surface expression of Gal and CD46 was measured by direct immunofluorescence using FITC-conjugated isolectin B4 from *Bandeiraea simplicifolia* (BS-IB4, Sigma), FITC-conjugated lectin from *Ulex europaeus* (UEA-I, Sigma), FITC-conjugated mouse anti-human CD46 Ab (Serotec, Raleigh, NC), and FITC-conjugated mouse IgG1 isotype control (Serotec). Cells were incubated for 30 min at 4 °C. Isotype-matched Ab or staining buffer alone (for BS-IB4, UEA-1) were used as negative controls.

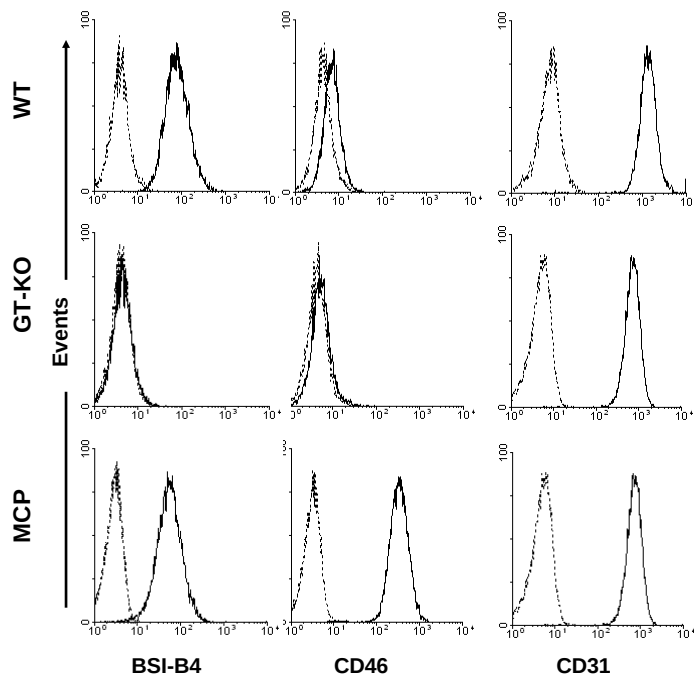


Figure 2.1-1 Surface expression of Gal and CD46 on PAECs.

PAECs from WT (upper panel), GT-KO (middle panel) and MCP (+/+) (lower panel) pigs were stained for BSI-B4, CD46 and CD31. Dashed lines – isotype control.

2.2 PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Peripheral human blood was drawn from healthy volunteers by venopuncture using pyrogen free syringes and needles combined with heparin for anticoagulant. Alternatively, cells were obtained from buffy coats. Blood was diluted in 1:1 PBS whilst buffy coats were diluted in 1:3 Peripheral blood mononuclear cells (PBMCs) from normal human blood were obtained using a standard Ficoll-Paque density gradient. After spinning

2000rpm for 20 minutes at room temperature, the middle layer of PBMCs were transferred to a new 50 ml tube for washing twice. The PBMCs were resuspended in 1ml of magnetic activated cell separation (MACS, Appendix 5) buffer; then incubated with 50 μ l mouse anti-human CD14 (Miltenyl Biotec, Auburn, CA) for 30 minutes on a roller. The monocytes were positively selected from PBMCs by using a magnetic cell separator (Miltenyl Biotec). The monocytes were counted on a hemocytometer.

2.3 PERPARATION OF PLATELETS

Venous blood was collected from healthy volunteers not taking anti-platelet drugs into tubes with anticoagulants of ethylenediaminetetraacetic acid (EDTA, 5 mM) or acid-citrate-dextrose (ACD). After centrifugation (10 minutes at 150g), the top two-thirds of platelet-rich plasma (PRP) was removed and centrifuged (8 minutes at 1400g). The pellet was washed with buffer (137 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4.1 mM $NaHCO_3$, and 5.5 mM glucose [pH 6.5]) containing prostaglandin E_1 (PGE_1 , Sigma) (120 nM). Platelets were centrifuged (150g for 5 minutes) to remove residual leucocytes (Panés et al., 2007). Cell counts were performed by contrast microscopy. Platelets were kept at 4°C during all the isolation steps after blood draw to avoid activation. The phenotypes and purity of platelets were evaluated by flow cytometry with anti-CD45 and anti-CD41Abs (BD Biosciences, Palo Alto, CA) to exclude leucocyte contamination.

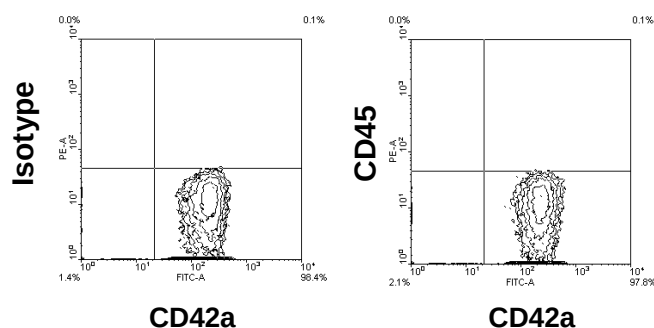


Figure 2.3-1 Phenotype and purity of isolated platelets.

Platelets were double stained with anti-CD42a and anti-CD45 Abs. The phenotype of platelets and the percentage of leucocytes (CD45 positive) were determined by flowcytometry.

2.4 PREPARATION OF SERUM AND PLASMA

Human or baboon plasma and serum were prepared after whole blood without/with anticoagulant agents were centrifuged at 1800 rpm for 5 minutes. Serum was heated at 56°C for 45 minutes to inactivate complement activity. Primate plasma and serum were stored at -80°C until use.

2.5 LACTATE DEHYDROGENASE (LDH) ASSAY

LDH (lactate dehydrogenase) assay was performed to evaluate complement-dependent cytotoxicity (CDC). 1×10^4 of PAECs were cultured in a 96-well, flat-bottom plate 24 hours prior to this assay. The cells were washed twice with PBS immediately before a series of concentrations of human serum was added. 100 μ l of 0.9% Triton X-100

(Promega, San Luis Obispo, CA) was used as positive control. After 4 hours incubation, a Cytotox 96 kit (Promega) was used to quantify the activity of cytoplasmic LDH in 50 μ l of cell lysate. Optical density values were measured on a microplate recorded at 492nm and the data analysis was carried out using Microsoft Excel.

Complement-mediated cytotoxicity = $\frac{OD_{492}(\text{total}) - OD_{492}(\text{background})}{OD_{492}(\text{Triton}) - OD_{492}(\text{background})} \times 100\%$

2.6 CH50 ASSAY

The CH50 assay (Dia Medix, Miami, FL) was used for determination of classical pathway complement activity in the fluid phase. The sensitized sheep erythrocytes were equilibrated at room temperature for one hour and resuspended with a vortex or by shaking vigorously. 5 μ l serum samples together with reference sera were added into the tubes containing the sensitized sheep erythrocytes. After one hour incubation at room temperature, the contents of the tubes were mixed by inverting them 3 -4 times. After centrifugation for 10 min at 1750 *g*, hemolysis was determined in the supernatant by measuring the absorbance of released hemoglobin at 412 nm, compared to the references.

2.7 ANTIBODY BINDING ASSAY

Binding of anti-porcine or anti-nonGal Abs to PAECs was measured by incubating 10^5 cells with 20% HI human serum or FACS buffer (control) for 30 minutes at 4°C. After incubation, the cells were washed twice in 2mL fluorescence activated cell sorting (FACS) buffer (Appendix 6) and centrifuged at 800g for 5 minutes. The supernatant was

discarded. To prevent non-specific binding, 10 μ L of 10% goat serum was added. Detection of IgM or IgG binding was performed by further incubating the serum with FITC-conjugated goat anti-human IgM (μ chain-specific) and IgG (γ chain-specific) (Invitrogen) for 30 minutes in the dark at 4°C. The samples were washed twice, and the cells resuspended with FACS buffer. Data acquisition was performed with BD™ LSR II flow cytometer (BD). Dead cells were excluded from the analysis by forward-scatter and propidium iodide (PI)(BD) staining. Measurement of mean fluorescence intensity (MFI) was performed on viable cells. Binding of IgM and IgG were assessed using relative MFI with the absence of serum (secondary Ab only) being scored as 1. The binding of anti-nonGal Abs was determined by incubating serum with GT-KO PAECs.

2.8 RECALCIFICATION CLOTTING ASSAY

2.8.1 Clotting assay in normal plasma

Functional TF activity of cells was determined by the recalcified clotting assay after 8-hour incubation with various stimuli. PAECs, monocytes, PBMCs or platelets were suspended in 50 μ L Tris-buffered saline (TBS, Appendix 7) and mixed with 100 μ L of normal human plasma (Haematologic Technologies, Essen Junction, VT) in glass tubes (Corning, Corning, NY). 100 μ L of 25 mM CaCl₂ in TBS were added and the tube incubated at 37°C in a water bath; the time for a fibrin clot to form was measured, during which time the tubes were continuously agitated by tilting. In the clotting assay with platelets, another 100 μ L phospholipid (Diagnostic Reagents) was added. The time for a fibrin clot to form was determined in triplicate for statistical analysis. There is one

drawback by using clotting assay to detect TF activity. First, even unactivated cells which do not express TF shorten clotting time compared to blank control (no cell). This shortening of clotting time between unactivated cells and blank control could result from the effects of other coagulation factors such as fgl2.

2.8.2 Clotting assay using FVII-deficient plasma

Since TF requires the involvement of FVII to initial the coagulation cascade, the recalcified clotting assay was repeated in FVII-deficient plasma to confirm that the shortening of clotting time was associated with the activity of TF. The shortening of clotting time in normal plasma will be normalized in FVII-deficient plasma if it is initialized by the presence of TF activity from the cells.

2.8.3 Clotting assay in FVII -deficient plasma with/without FVII

2.8.3.1 Dosage of FVII in the recalcified clotting assay

In these assays, TF-dependent thrombin generation required the participation of factor VII (FVII, Haematologic Technologies). The clotting time was shortened when FVII was added to this system. The amount of FVII was determined by adding a series of concentrations of FVII in the clotting assay with TNF α -activated PAECs. Therefore, 0.05 U/10ul of FVII was used in the further experiments (Figure 2.8-1).

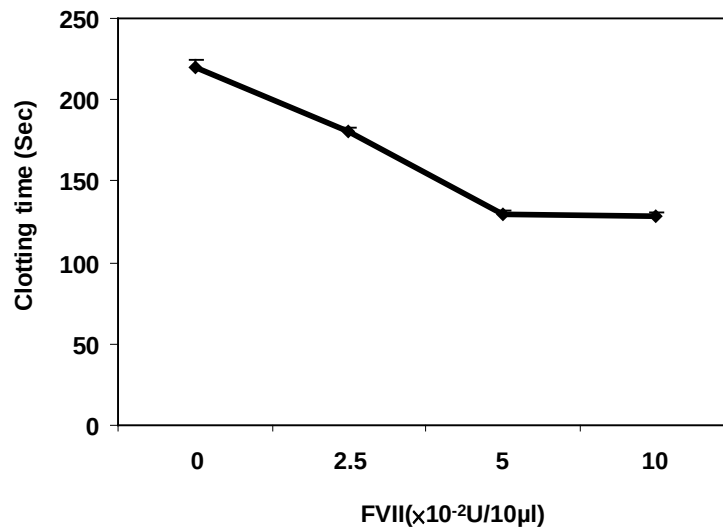


Figure 2.8-1 The dosage of FVII was determined in FVII-deficient plasma recalcified clotting assay with the addition of FVII.

The recalcified clotting assays were performed in FVII-deficient plasma by using 1×10^5 PAECs, which had been activated by $\text{TNF}\alpha$ for 8 hours. The clotting times were measured when a series of diluted concentrations of FVII were added.

2.8.3.2 Standard curves of TF activity

The procedure was repeated with the addition of FVII (in a separate assay) and the activity of TF was determined by the ratio of the clotting times measured with/without FVII. The clotting times were determined in triplicate and results, were quantified from a standard curve prepared by a series of dilutions of soluble recombinant human TF (R&D, Minneapolis, MN) and expressed as a procoagulant activity equivalent to ng human TF per 1×10^5 PAEC, 1×10^5 PBMCs or 2×10^6 platelets (Figure 2.8-2).

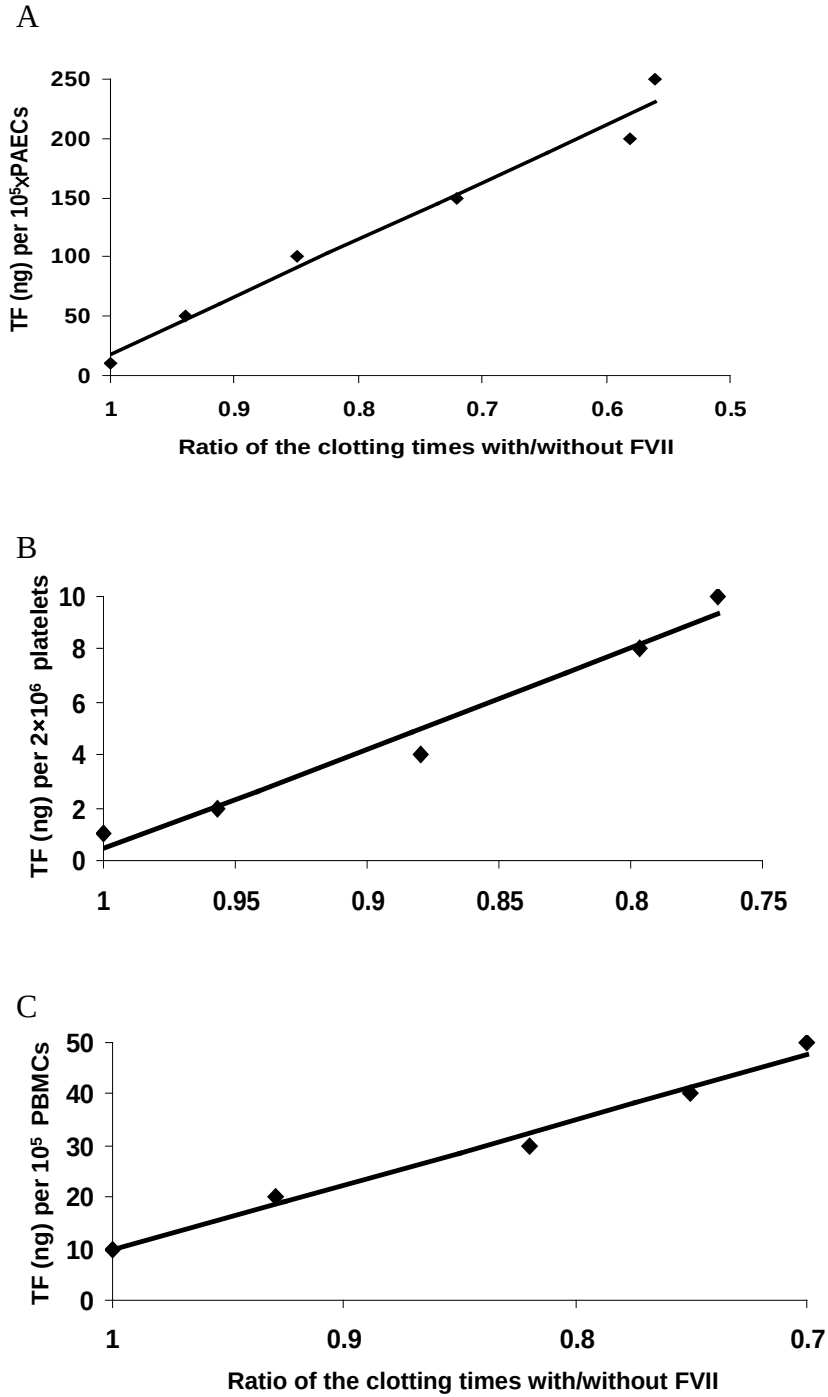


Figure 2.8-2 Standard curves of TF activity in FVII-deficient plasma clotting assay.
PAECs (A), platelets (B) and PBMCs (C).

2.9 FLOWCYTOMETRY

Flow microfluorometry of cells was performed with an argon laser emitting 488 nm and a quartz flow cell. For non-suspension cells (ECs), the cells were harvested by trypsin-EDTA, and then maintained at 4°C for all subsequent handling. The cells were washed twice, then counted and assessed for viability. The cells were pelleted again by centrifugation for 5 minutes at 1800 rpm at 4°C and resuspended in cold FACS buffer containing fluorescently-conjugated primary Abs or non-conjugated primary Abs. If unlabeled primary Abs were used, the cells were stained again with fluorescently-conjugated secondary Abs after 30 minute incubation with primary Abs and washing twice. The primary and secondary Abs were listed in Table 2.9-1,2.

Antigens	Clones (isotype)	Sources
CD31 PE	TLD-3A12 (mouse IgG1 κ)	BD Pharmingen
CD42a FITC	ALMA,16 (mouse IgG1)	BD Pharmingen
CD45RA PE-Cy TM 5	5H9	BD Pharmingen
CD46 FITC	MEM-258 (mouse IgG1)	Serotec
CD62p FITC	AC1.2 (mouse IgG1)	BD pharmingen
CD62P/E (human)	1.2B6 (mouse IgG1)	Serotec
CD62P (human)	AK4 (mouse IgG1 κ)	eBioscience
CD62P (pig)	12C5 (mouse IgG)	Gift from Professor Haskard
CD106	10.2C7 (mouse IgG)	Gift from Professor Haskard (Imperial College)
Gal (BS-IB4) FITC		Sigma
Gal (UEA-1) FITC		Sigma
IgG (γ chain-specific)		Invitrogen
IgM (μ chain specific)		Invitrogen
Mouse IgG1 isotype	X40	BD Pharmingen
Mouse IgG1 FITC	clone W3/25	Serotec
Mouse IgG1 PE		Serotec
Mouse IgG2b	X40	BD Pharmingen
Sheep IgG		Affinity Biologicals
TF	Goat polyclonal IgG	Affinity Biologicals
TFPI	Rabbit polyclonal IgG	American Diagnostica
vWF	F8/86 (mouse IgG1)	Dako

Table 2.9-1 Primary antibodies for flowcytometry.

Determinants	Conjugates	Sources
Anti-mouse IgG (goat)	FITC	Sigma
Anti-sheep IgG (donkey)	FITC	Sigma

Table 2.9-2 Secondary conjugated antibodies for flowcytometry.

2.10 QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

2.10.1 Preparation of RNA from cells and tissues

Total RNA was extracted by using the 500 µl Trizol reagent (Invitrogen) from the cells (2×10^5 PAECs, 2×10^6 PBMC and 2×10^7 platelets) and tissue (native or transplanted heart, kidney and liver), which were grind to particles on dry ice as small as possible. 100 µl of chloroform (Sigma) was added to the suspension, which was vortexed for 15 sec and incubated on the ice for 10 minutes prior to centrifugation at 14,000g for 10 minutes. The upper layer containing RNA was mixed with equal volume of isopropanol (Sigma) at 4°C for 10 minutes. The RNA was precipitated by centrifugation at 14,000 g for 10 minutes. The RNA pellet was washed once with 75% ethanol and suspended in 1µl gel, showing 3 RNA bands (no evidence of laddering indicative of RNA degradation). RNA content was measured using 260/280 UV spectrophotometer.

2.10.2 RT-PCR

RT-PCR was performed by a two step technique. Complementary DNA (cDNA) was prepared from RNA. Three micrograms (3µg) of total RNA in 10µl from each sample and 1µl oligo dT (10µg/µl) (Invitrogen) were heated to 68°C for 4 minutes and immediately cooled to 4°C for 15 minutes. The reaction matrix containing 4µl 5x first strand buffer (250mM Tris-HCl, 375mM KCl, 15mM MgCl₂) (Invitrogen), 2µl of 100mM dithiothreitol (DTT) (Invitrogen), 2µl of RNAase free water, 1µl of 10mM dNTPs (Invitrogen) and 1µl of Superscript III (Invitrogen) was added to RNA to generate first-

strand cDNA. The sample was incubated for 1 hour at 42°C, and heated to 72°C for 15 minutes to stop the reaction. The synthesized cDNA was stored at -20°C.

2.10.3 Quantitative PCR

PCR mixture was prepared using 8 µl cDNA (0.15µg/µl), 10µl SYBR Green PCR Master Mix (Applied Biosystems) and 1 µl of sense and anti-sense primers. Primers for porcine and baboon TF were purchased from Invitrogen and are listed in Table 2.10-1. The primers were design to span at least one intron of the genomic sequence to avoid genomic DNA contamination. The sequences of the above primers were analyzed in triplicate. Thermal cycling conditions were 10 minutes at 95°C to activate the Amplitaq Gold DNA polymerase, followed by 40 cycles of 95°C for 15sec and 60°C for 1minute on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Using the manufacturer's software, real-time PCR data were plotted as the ΔRn fluorescence signal versus the cycle number. The threshold cycle was defined as the cycle number at which the ΔRn crosses this threshold. The expression of each gene was normalized to actin mRNA content and calculated relative to control using the comparative CT method.

Primers	Sequences
Baboon TF	Sense 5'-TGCTTTTACACAGCAGACACAGAGT-3'
	Anti-sense 5'-AAGACCCGTGCCAAGTACGT-3'
Baboon β actin	Sense 5'-TGGAAGAATGCGGCTCATATT-3'
	Anti-sense 5' TACTATCCAATCCTAGAAAGAACATG-3'
Porcine TF	Sense 5'- TTTACCAACTCGCCCCCCTTC -3'
	Anti-sense 5'- AATGTGCCGTTCACCCTGACTAAG -3'
Porcine β actin	Sense 5'- CTCGATCATGAAGTGCGACTG -3'
	Anti-sense 5'-GTGATCTCCTTCTGCATCCTGTC-3'

Table 2.10-1 RT-PCR primers of TF and β actin. All primers are known to span intron.

2.11 IMMUNOFLUORESCENCE STAINING

Tissues were harvested, immersed in OCT compound (Miles, Elkhart, Indiana), snap-frozen in liquid nitrogen and stored in -80°C. Sections were cut on a Cryostat and dried for one hour at room temperature. 4 μ m-frozen sections were prepared on gelatin-coated slides at the Department of Pathology in the Thomas E. Starzl Transplantation Institute. The samples were kept at room temperature for 30 minutes and fixed in cold acetone (Sigma) for 10 minutes. After washing with 0.5% PBS, slides were transferred to 5% goat serum (Sigma) for 20 minutes at room temperature to prevent nonspecific Ab binding. Then the slides were stained with primary Abs for 60 minutes at room temperature or overnight at 4°C in the dark; then after washing $\times$ 3, appropriate secondary Abs were stained for another 60 minutes at room temperature. DAPI (Vector Laboratories, Burlingame, CA) was used for nuclear DNA staining. Finally, the slides were fixed with 4% paraformaldehyde and prepared by coverslips with gelvatal. The slides were examined in a fluorescent microscope (Olympus, CenterValley, PA). The primary and

secondary Abs used in immunofluorescent staining are summarized in Table 2.11-1.

Determinants	Clones (Isotype)	Sources
CD31(pig)	mouse IgG	Atigenix American
CD41 (human)	rabbit polyclonal IgG	Abcam
CD45 (human)	NPH	BD Pharmingen
CD106 (human)	10.2C7(mouse IgG)	Gift from Professor Haskard
Fibrin (human)	Sheep polyclonal	Affinity Biologicals
TF (human)	Sheep polyclonal	Affinity Biologicals
TF (pig)	rabbit polyclonal IgG	Gift from Dr Vladimir Bogdanov
Secondary	Conjugation	
Donkey anti-sheep IgG	Cy2	Jackson ImmunoResearch
Goat anti-mouse IgG	Cy 3	Jackson ImmunoResearch
Donkey anti-rabbit IgG	Cy 5	Jackson ImmunoResearch

Table 2.11-1 Primary and secondary antibodies for immunofluorescence staining

2.12 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

The histological and immunohistochemistry examinations of all tissues were performed by Dr Bertha Garcia in the Department of Pathology, University of Western Ontario, London, Canada. For conventional histology, tissues were fixed in 10% formalin and embedded in paraffin. Four-micron (4µm) sections were stained with hematoxylin and eosin (H&E) and with martius-scarlet-blue (MSB) for light microscopy. Immunohistochemistry staining for IgM, IgG, C3, C4d, and fibrin was performed on paraffin sections.

2.13 *IN VITRO* CULTURE SYSTEM

2.13.1 Optimization of culture system

PAECs or HAECs adherent to a culture flask were pre-incubated for 8 hours with fresh or HI human serum (5%), human platelets (5×10^7 /ml), monocytes (5×10^5 /ml), or combinations of all three. Five percent (5%) human plasma was selected because this concentration resulted in near-saturation of IgG and IgM binding to PAECs by flowcytometry, and caused <10% CDC (Figure 2.13-1). Human serum, human platelets and monocytes were isolated from blood type A volunteer donors to minimize the effect of ABO-incompatibility. After coculture, human monocytes or platelets were collected from supernatants, and PAECs were harvested by prewarmed 0.5% trypsin at 37°C for flow cytometry and recalcified clotting assay analysis, respectively.

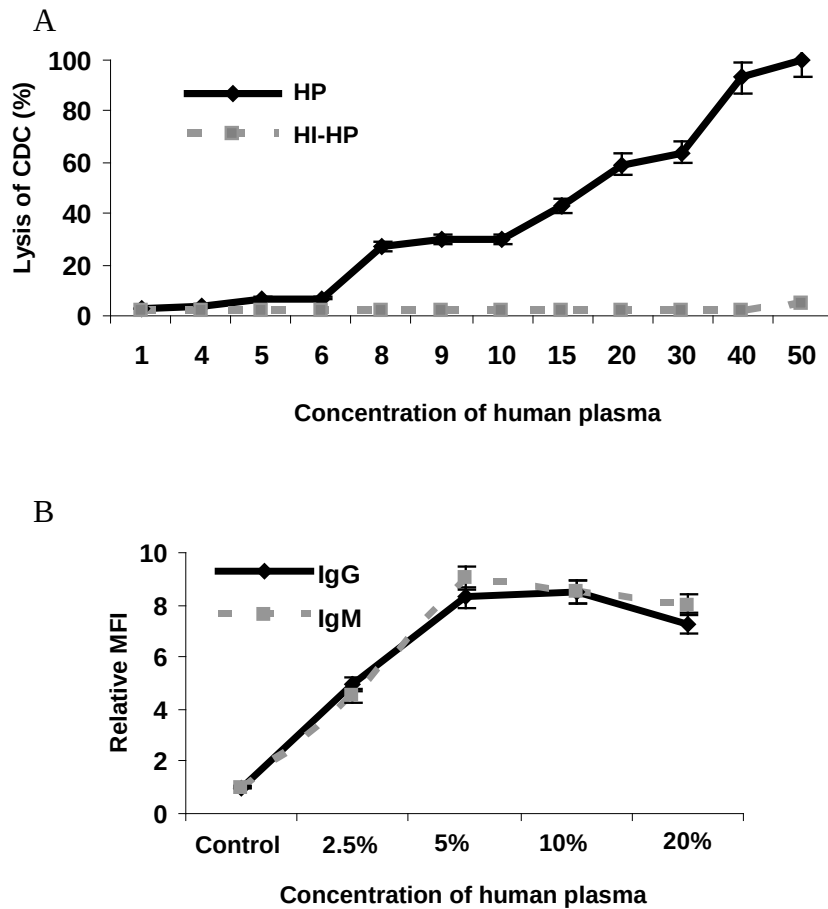


Figure 2.13-1 Complement dependent cytotoxicity and antibody binding determine optimal concentration of human plasma in the culture system.

- A. The PAECs were incubated with a series of concentrations of fresh or heat-inactivated (HI) human plasma (HP) for 4 hours. The CDC of human serum was determined by LDH lytic assay.
- B. The PAECs were incubated with a series of concentrations of HI human plasma for 30 minutes at 4 °C. IgG and IgM binding were determined by flowcytometry.

2.13.2 Transwell culture system

To dissect the molecular interactions between adhesive PAECs and platelets or monocytes, a transwell system was used to separate the two components and determine the role of cell-to-cell contact. Adhesive PAECs were cultured in the lower chamber; platelets and monocytes/PBMCs were incubated in the upper chamber. The conditions of the culture system were the same as in the non-transwell culture system.

2.13.3 Neutralizing antibody experiments

In some experiments, the cells involved in the culture system were pre-incubated with a series of diluted concentrations of neutralizing Abs for 30 minutes at 4°C in the same medium as used in the culture system. The neutralizing Abs are listed in the Table 2.13-1. Then the cells were incubated as previously described without washing.

Determinants	Clones (Isotype)	Sources
CD106 (human)	10.2C7 (mouse IgG)	Gift from Professor Haskard
CD162 (human)	PL1 (mouse IgG1 κ)	MBL International
CD41 (human)	PM6/288 (mouse IgG1)	Serotec
CD42b (human)	Ak2 (mouse IgG1)	Serotec
CD62P (human)	AK4 (mouse IgG1 κ)	eBioscience
CD62P (pig)	12C5 (mouse IgG)	Gift from Professor Haskard
CD62P/E (human)	1.2B6 (mouse IgG1)	Serotec
Clk-1 (human)	Goat polyclonal IgG	Santa Cruz
IgG Fab (human)	Goat IgG	Sigma
PAR-1 (human)	Goat polyclonal IgG	R&D system
SLA I	1E3 (mouse IgG1)	BD
SLA II (SLA-DR)	1053h2-18-1(mouse IgG2a)	BD
TF (human)	Sheep polyclonal IgG	Affinity Biologicals
vWF (human)	1.B 690 (mouse IgG1)	Santa Cruz

Table 2.13-1 Neutralizing antibodies.

2.14 IN VITRO PLATELET AND PBMC AGGREGATION MODEL

Platelets play a key role in mediating inflammatory and thrombotic responses during health and disease. Once activated, circulating platelets bind to leucocytes, predominantly monocytes, forming platelet-leucocyte aggregates. Importantly, we observed that platelet-leucocyte aggregates dramatically increased during development of consumptive coagulopathy in pig-to-primate organ transplantation (Chapter 7).

Therefore, an *in vitro* model was set up to clarify the interaction between platelets and PBMCs by the aggregates. Platelets and PBMCs were isolated from the same individual primates. 5×10^5 PBMCs and 5×10^6 platelets (1:10 ratio) were co-cultured with medium (control), HAECs (allograft control) and PAECs in 6 well plates with 2ml culture medium. The cells were stained by CD45RA as a PBMC marker and CD42a as a platelet marker. The percentage of CD42a-positive cells in CD45RA cells were designated as platelet and PBMC aggregates.

2.15 ANIMAL MODELS

Baboons (*Papio* species, Division of Animal Resources, Oklahoma University Health Sciences Centre, Oklahoma City, OK, and the Southwest Foundation for Biomedical Research, San Antonio, TX) weighing 6-10kg and of known ABO blood type, were recipients. Pigs of blood group O (nonA), weighing 10-20kg (Revivicor, Blacksburg, VA), served as sources of xenograft organs, and were generated by nuclear transfer/embryo

transfer from modified fibroblasts from Large White/Landrace/Duroc cross-breed pigs. All pigs were negative for expression of cytomegalovirus (CMV).

Pigs from 4 sources, namely (i) wild-type (WT) or (ii) GT-KO pigs, or (iii) pigs transgenic for the complement-regulatory protein, CD46, or (iv) TFPI. The WT, GT-KO, and TFPI transgenic pigs were of Large White/Landrace/Duroc cross-breed, but were not from identical clones; however, the only major difference was in the absence of Gal antigens in the GT-KO pigs (Phelps et al., 2003) or the expression of TFPI. The differences of DNA sequences between wild type of GT-KO pigs were presented in Figure 2.15-1 Sequencing analysis of the α 1,3GT gene from wild-type pig cells and DKO porcine fetal fibroblasts.. The CD46 transgenic pigs were derived from a different herd of Large White pigs (Loveland et al., 1993). Constructs of TFPI encoding three human Kunitz domains of TFPI-CD4-P-selectin, and in vitro characterization after expression on endothelial cells have been described previously (Chen et al., 1999a; Chen et al., 1999b; Chen et al., 2004b; Riesbeck et al., 1997). A human CD31 promoter was generated and cloned into the vector for microinjection .

The pig-to-baboon organ transplantations included kidney, liver and aortic patch grafts. The aortic and kidney transplantation were organized by Dr Cooper and Dr Ezzeelrab; the liver was organized by Dr Cooper and Dr Ekser.

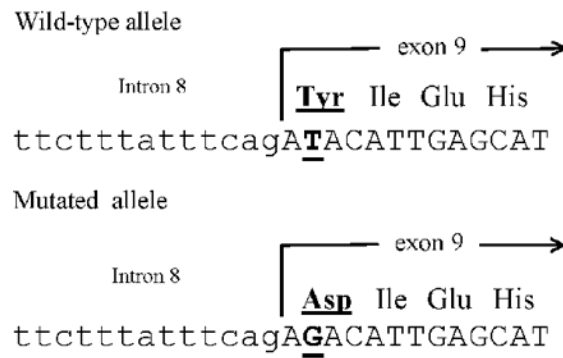


Figure 2.15-1 Sequencing analysis of the α 1,3GT gene from wild-type pig cells and DKO porcine fetal fibroblasts.

Small letters and capital letters denote intron and exon sequences, respectively. Underlined capital letters indicate the nucleotide where the point mutation occurred. Amino acids deduced from the correspondent mutated and wild-type DNA sequences are underlined (Adapted from Phelp CJ, Science, 299:411, 2003.)

2.15.1 Preoperative preparation of baboons

In the pig-to-primate xenotransplantation model, the baboons were quarantined for at least one month to exclude infective diseases. Three weeks before operation, the baboons were shaved to remove hair on the truck and upper arms and were put in jackets. After one-week training, a tether was fixed to the jacket and connected to the cage for another a week training. Approximately 7 days pretransplantation, intravascular catheters were inserted into the right internal and external jugular veins and carotid artery under general anesthesia, allowing drug and fluid administration and blood withdrawal. Antibiotics (cefazolin) and analgesia were administered twice a day for 3 days and an anti-ulcer drug (histamine2 –blocker, famotidine 0.25 mg/kg) was given twice a day.

2.15.2 Donor organ harvest

Under full inhalational anesthesia, the surgery was begun with a long midline laparotomy, usually performed by Dr Hara and Dr Echeverri. After heparinization (300 units/kg, IV), 1 liter of cold UW solution was perfused via the abdominal aorta, allowing the effluent to drain from the hepatic veins after division of the inferior vena cava. The organs, such as liver and kidneys were covered with cold saline and ice slush to cool them externally while the perfusate was running. The donor organs such as kidney, liver were procured by dividing the adjacent vessels and stored in ice-packed UW solution until transplanted. The donors were euthanized by exsanguination under anesthesia.

2.15.3 Aortic patch transplantation

A long midline laparotomy was performed as a sterile procedure and the abdominal aorta was isolated. The aorta was opened longitudinally between the origin of the inferior mesenteric artery and the bifurcation. An oval-shape aorta patch, which was harvested from internal carotid or iliac arteries of a GT-KO pig were sutured under the side clamp of aorta (Figure 2.15-2) When all bleeding was meticulously stopped, the abdominal incision was closed layer by layer with interrupted sutures to minimize the risk of dehiscence. This model was used to test the efficacy of different combinations of immunosuppressants compared with no immunosuppressant. At the same time, the baboons in the control group without immunosuppression were sensitized by grafts from GT-KO pigs and conferred therefore high titres of anti-nonGal Abs.

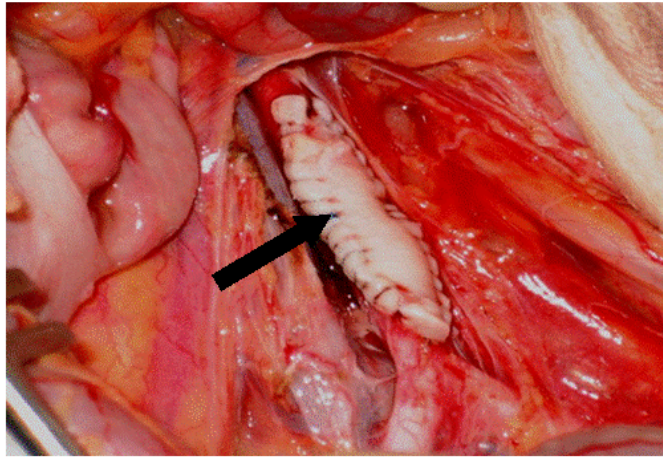


Figure 2.15-2 Aortic patch transplantation.

Aortic patch from GT-KO pig (Arrow) was implanted into lower abdominal aorta of a baboon.

2.15.4 Kidney transplantation

Through a midline laparotomy, both kidneys were isolated and removed by ligation and division of renal arteries, veins and ureters. The porcine donor kidney was implanted by renal artery-aortic, renal vein-caval anastomoses and ureterocystostomy. Since both native kidneys were removed, all urine excretion was from the implanted xenograft, which was one of the parameters of renal function. This model was performed to test the benefit of organs from genetically-modified pigs, such as GT-KO, MCP (+/+) and GT-KO with MCP (+/+).

2.15.5 Liver transplantation

Through a midline laparotomy, the native liver was removed by division of the common bile duct, hepatic artery, portal vein and inferior vena cava. The donor liver was

implanted by cavo-caval anastomoses for outflow reconstruction, subsequently portal-portal and hepatic artery to celiac trunk anastomosis and choledocho-choledochostomy for biliary reconstruction. The model was performed to examine the possibility of a xenoliver transplantation becoming a bridging procedure to allotransplantation for patients with acute hepatic failure, and to investigate the benefit of donor organs from GT-KO and GT-KO/ MCP (+/+) pigs compared to WT pigs.

2.15.6 The definition of consumptive coagulopathy

Although consumptive coagulopathy has been recognized for a long time in xenotransplantation, the distinct definition and clinical manifestations remain unclear. Several coagulation profiles such as fibrinogen, thrombin-antithrombin, D-dimer and PT/INR, were used to detect consumptive coagulopathy (Cozzi et al., 2004; Chen et al., 2005).

During AHXR, many recipients develop consumptive coagulopathy, which presents with massive consumption and exhaustion of coagulation proteins and activation and aggregation of platelets. In this study, consumptive coagulopathy was defined when the following signs occurred: 1. unexplained thrombocytopenia and anemia; 2. decreased serum level of fibrinogen; 3. baboons being unwell clinically and requiring euthanasia; 4. overt bleeding.

2.15.7 Animal groups

Organ transplantation was divided into 4 groups. A baboon-to-baboon liver allotransplantation was used as an allograft control, and designated as Group I (liver, n=1). A HAR model was developed by using WT pig-to-baboon xenograft without any immunosuppressant as Group II; (liver, n=1 and kidney, n=1). Non-HAR models were performed by transplanting organs from WT, GT-KO or GT-KO/MCP (+/+) pigs into baboons with or without immunosuppressants to avert HAR and were designated as Group III (kidney, n=8) and Group VI (liver, n=4). The types of grafts and immunosuppression are described in Table 7.2-1. Four baboons that underwent liver allotransplantation (2) and xenotransplantation (2) were excluded because their survivals were less than 24 hours due to severe hypotension or hypoxic injury during the operation. Immunosuppressive protocol

The recipient baboons received either no immunosuppressive therapy (n=4), MMF-based immunosuppressant for kidney grafts (n=5), or tacrolimus-based immunosuppressant for liver transplantation (n=5). Thymoglobulin, low-dose anti-CD154mAb (with a human anti-human CD154mAb (ABI193) generously provided by NovartisPharma, Basel, Switzerland) and mycophenolate mofetil (MMF) were routinely used in kidney transplantation groups with immunosuppressants. CVF was used in one WT baboon (B3907) but not in other baboons when the grafts were from pigs that were transgenic MCP. In liver transplantation, thymoglobulin, tacrolimus and MMF were given. Methylprednisolone was administered in both groups, then tapered. The details of dosage and timing of immunosuppressants are summarized in Table 2.15-1,2.

Induction Therapy	Dose	Duration
Thymoglobulin	1-10mg/kg	day -3 and -1
Cobra venom factor	100 units/day	days -1 to 3
Maintenance Therapy	Dose	Duration
Anti-human CD154mAb	25mg/kg	days -1, 0, 4, 7, 10, 14, and then
	20-25mg/kg	every 5 days (to maintain a blood trough level >400µg/mL)
Mycophenolate mofetil	25-110mg/kg/day	from day -2 (to maintain a constant blood level of 3-5µg/mL)
Methylprednisolone	4 - 0.5mg/kg	from day 0 (tapering)
Supportive therapy	Dose	Duration
Prostacyclin	20ng/kg/min	days 0-7
Dopamine	2-3µg/kg/min	day 0
Ganciclovir	5mg/kg/day	from day -5
Famotidine	0.25mg/kg/x2/day	from day -5
Heparin	5-50U/kg/h	from day 0 (to maintain PTT at 100-150 sec from day 3)

Table 2.15-1 Immunosuppressive and supporting therapy in kidney xenotransplantation.

*All therapy was given intravenously, PTT = activated partial thromboplastin time

Induction therapy	Dosage	Duration
Thymoglobulin	1-10 mg/kg IV	1-10 mg/kg IV
Maintenance therapy	Dosage	Duration
Tacrolimus	0.05-0.2 mg/kg twice a day IM	Starting from -2
MMF	110 mg/kg continuous IV	Starting from -4
Steroid	10 mg/kg/day	Starting from 0 and taper

Table 2.15-2 Immunosuppressive protocol for liver xenotransplantation.

IV: intravenous, IM: intramuscular

2.15.8 Postoperative medication and supportive care

All animals received prophylactic intravenous (iv) antibiotics for the first 3 post-transplant days and some animals received daily ganciclovir for prophylaxis of CMV infection. Because consumptive coagulopathy can develop in baboons with pig organ grafts and in view of the published association of some anti-CD154mAbs with thromboembolic complications, heparin was begun on day 0 (day of Transplantation) and gradually increased in an effort to maintain the activated partial thromboplastin time at approximately 150sec from day 3. However, this goal was not always achieved. Washed baboon red blood cells were administered when necessary to maintain the hematocrit >20%.

2.15.9 Monitoring of recipient baboons

Blood cell counts, chemistry, and coagulation parameters were measured initially daily and subsequently at several time-points each week or weekly by standard methods. The serum trough level (immediately before the next dose) of the anti-CD154mAb was monitored by ELISA (maintained at $>400\mu\text{g/mL}$ in baboons). Blood cultures were performed when indicated by clinical features suggesting infection.

2.15.10 Euthanasia of animals

For the welfare of the animals, they were euthanized with sodium pentothal in the necropsy room. Blood and tissue samples were harvested during euthanasia. In the studies related to this thesis, the baboons were euthanized when they developed untreatable consumptive coagulopathy or the grafts failed.

2.16 HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS

Whole blood was collected in 3.2% trisodium citrate anticoagulant solution. All hematologic and biochemistry tests were performed at the Central Laboratory or Transfusion Medicine Diagnostics Coagulation Reference Laboratory of University of Pittsburgh Medical Centre. Both automated and manual assays were utilized in this study. Automated assays were performed using a Behring Coagulation System (BCS; Dade Behring, Newark, DE) and an ACL 3000 (Instrument Laboratories, Miami, FL).

2.17 STATISTICAL ANALYSIS

Statistical analysis was performed using Student's t test in SPSS, 11th version (Chicago, IL) software. For any *in vitro* work shown in graphical presentation, the data were presented with mean $\pm$ SE (standard error) based on 3 independent experiments. $P < 0.05$ was considered significant. The mean and standard error were calculated using Microsoft Excel functions (Microsoft Corporation, Redmond, WT).

2.18 ETHICAL APPROVAL

All animal care was 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 were approved by the Institutional Animal Care and Use Committee in the University of Pittsburgh.

CHAPTER 3

TF EXPRESSION ON PAECs, PLATELETS

AND MONOCYTES *IN VITRO*

3.1 INTRODUCTION

The Ab-mediated immunologic barrier between primates and pigs hinders the success of xenotransplantation. Several strategies have been developed to overcome HAR and prolong graft survival (Cooper et al., 2007). Nonetheless, AHXR ensues and leads to intravascular thrombosis.

For example, transplanting hearts from GT-KO pigs (Phelps et al., 2003) into baboons prolonged median survival to 78 days, but eventually all grafts succumbed to ischemic necrosis from thrombotic microangiopathy (TM) (Kuwaki et al., 2005). Nevertheless, the pathology in these grafts was different from typical AHXR and revealed microvascular thrombosis in arterioles, capillaries, and venules, with only rare interstitial mononuclear cells. Whether these changes resulted from low-grade humoral rejection or non-immunologic factors, such as coagulation dysregulation, remains uncertain.

TF binds FVII/VIIa, and the complex TF-FVIIa activates FX and FIX to initiate coagulation (Mackman, 2004; Morrissey, 2001). ECs and monocytes constitute the main origins of TF, as shown in inflammation and sepsis models (Keller et al., 2003; Warr et al., 1990). Microparticles shed from EC, or monocytes, are the main source of circulating TF, and transfer TF to platelets (Giesen et al., 1999; Monroe and Key, 2007). Recently, platelets have been shown to be capable of synthesizing and expressing functional TF (Schwartz et al., 2006).

The importance of TF as the initiator of thrombosis after xenotransplantation has not been formally studied. *In vivo* studies demonstrated that expression of TF was up-regulated in necrotic xenografts (Ierino et al., 1998; Nagayasu et al., 2000; Shimizu et al., 2005). The expression of TF on PAECs was up-regulated by activated platelets or complement by XNA (Gollackner et al., 2004; Xu et al., 2001). These studies suggested TF as an initiator of xenograft thrombosis.

However, the origins of TF and the interaction between PAECs, human monocytes and platelets are not fully understood. In this study, we developed an *in vitro* model to attempt to elucidate the interactions between PAECs and human monocytes and platelets in terms of expression of TF, and we attempted to demonstrate that TM is initiated by TF.

3.2 RESULTS

3.2.1 The characteristics of anti-human TF antibody

Upregulation of cell surface TF expression was detected on PAECs after activation by TNF- α (10 ng/ml, Sigma) by FACS analysis using the sheep anti-human TF Ab (Figure 3.2-1A). These cells promoted shorter clotting times of recalcified normal plasma compared to control unactivated PAECs (Figure 3.2-1B), however the Ab failed to inhibit this difference, indicating that although it detected TF expression on the surface but it did not functionally inhibit porcine TF-initiated coagulation. The shortening of clotting involved with activated PAECs resulted from the effect of TF since it was normalized when performed in FVII-deficient plasma (Figure 3.2-1C). In contrast, it was able to inhibit the activity of human TF on a TF-positive control human breast cancer cell line (Figure 3.2-1B). Therefore, this Ab was used to differentiate the origin of TF, either from human or pig.

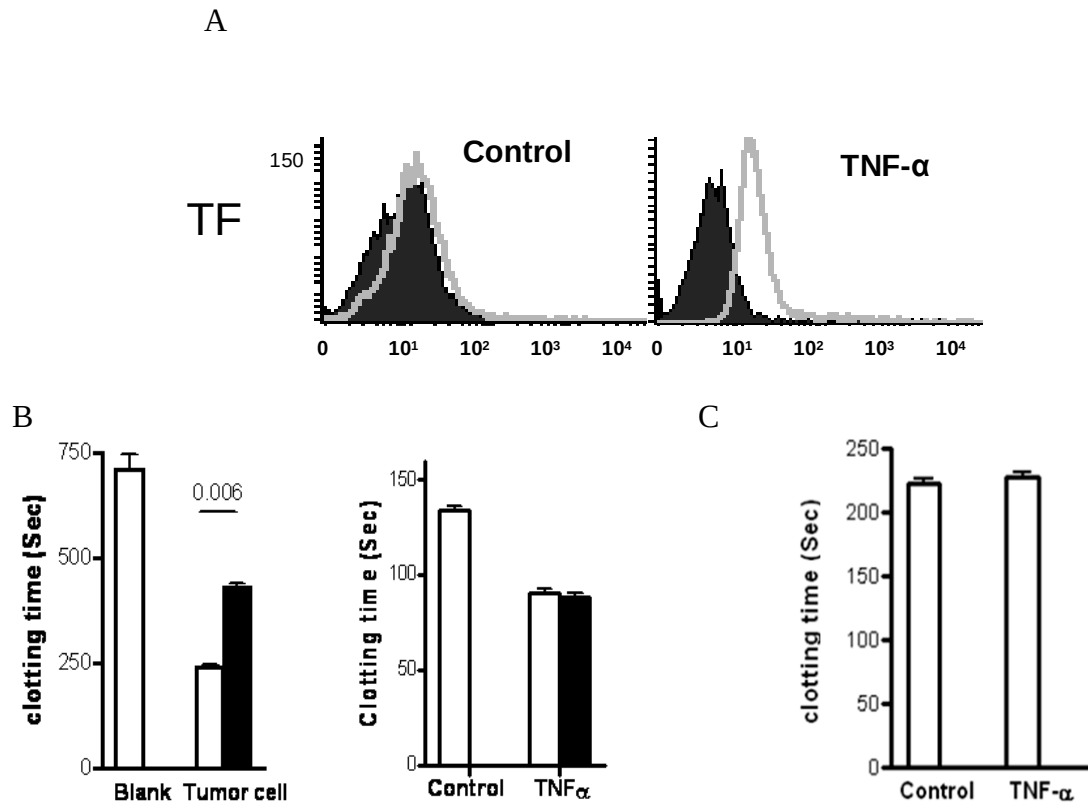


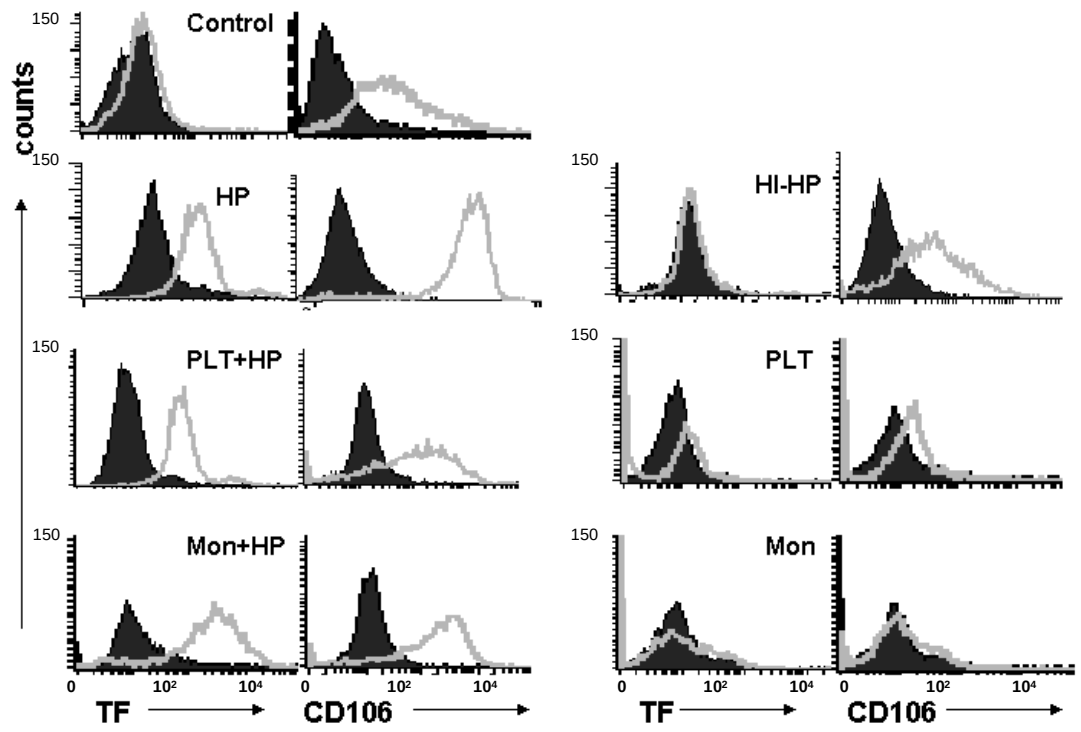
Figure 3.2-1 The characteristics of sheep anti-human TF antibody

- A. PAECs activated by TNF- α (10ng/ml) for 8 hours induced porcine TF activity measured by flow cytometry using the sheep anti-human TF Ab (open) or an isotype control (black).
- B. Clotting times of recalcified normal human plasma. Left panel: results without exposure to cells (control, blank) or after exposure to a human TF-positive breast cancer cells. Right panel: results following no activation (control) or TNF α activation of PAECs. (Open bars - no additional sheep anti-human TF Ab. Closed bars - sheep anti-human TF Ab incubated with cells for 30 minutes prior to the clotting assay.)
- C. Clotting time of recalcified FVII-deficient plasma involved with activated PAECs

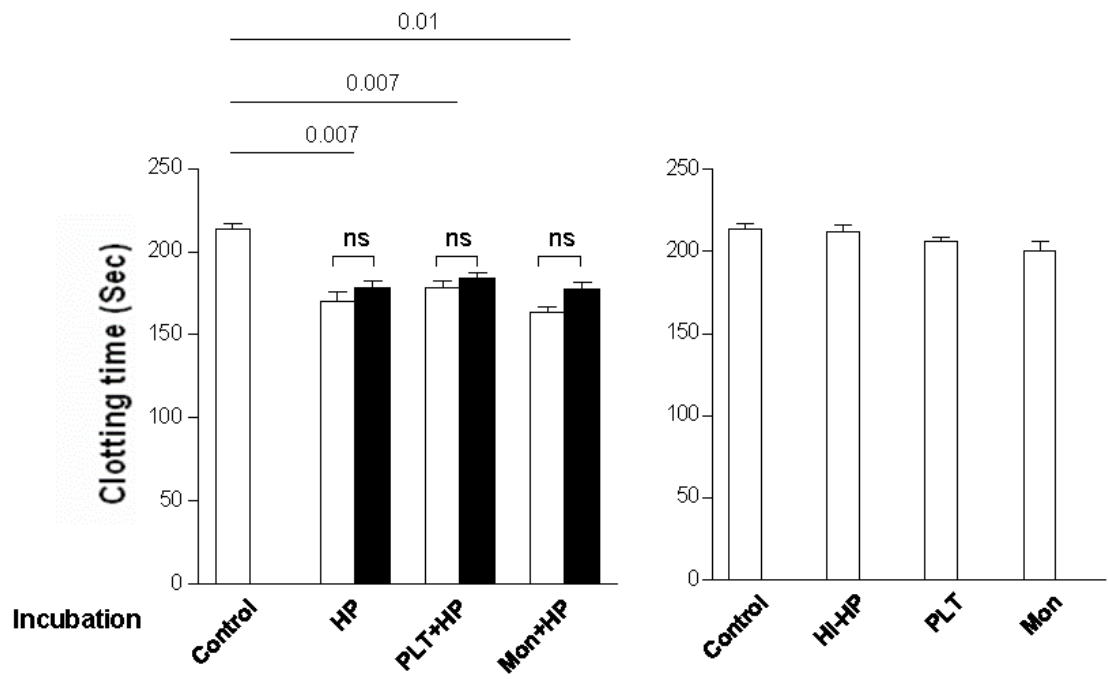
3.2.2 Procoagulant activity of PAECs

After incubation with human plasma, human platelets, human monocytes, or combinations of all three, expression of TF and VCAM-1 (CD106; as an activation control) on PAECs was analyzed by flowcytometry. Untreated PAECs were TF-negative and VCAM-1-positive. Prior incubation with human plasma induced TF and VCAM-1 expression (Figure 3.2-2A). When platelets or monocytes were included in the incubation step with human plasma, they had no additional impact on TF or VCAM-1 expression (Figure 3.2-2A). Incubation of PAECs with platelets or monocytes alone in the absence of human plasma did not induce TF or VCAM-1 expression (Figure 3.2-2A). However, HI human plasma resulted in no induction of TF or VCAM-1. The observed induction of TF antigen on the surface of PAECs was confirmed by increased TF activity as evidenced by a shortening of clotting times in a normal plasma recalcified assay (Figure 3.2-2B). The shortening of the clotting time was shown to be TF-dependent since the PAECs did not shorten the clotting time of FVII-deficient plasma (Figure 3.2-2C). The failure of the anti-human TF Ab to inhibit the clotting activity demonstrates this activity is porcine TF and not human TF derived from the human plasma (Figure 3.2-2B).

A



B



C.

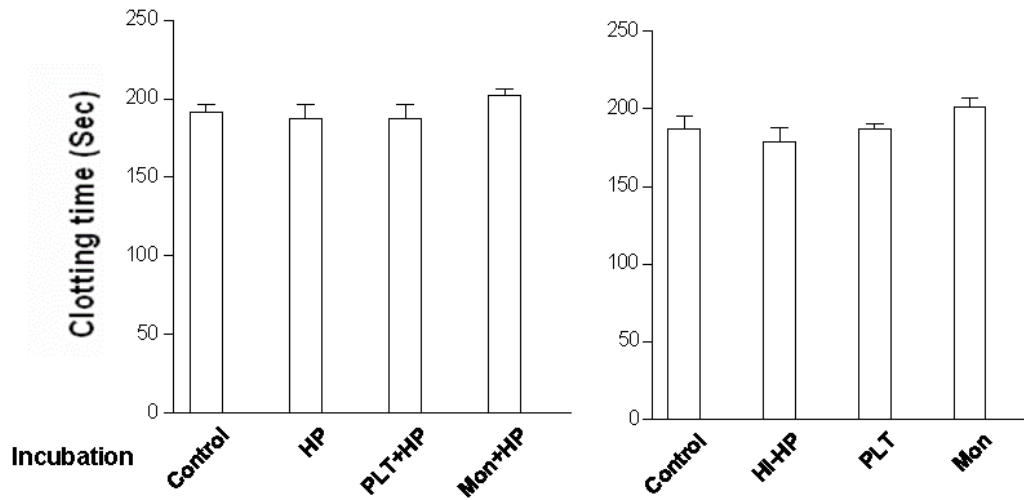


Figure 3.2-2 PAECs express TF activity dependent on the presence of complement.

PAECs were co-incubated with various stimuli for 8 hours, and studied by flow cytometry and the recalcified clotting assay performed using both normal and FVII-deficient plasma. Addition of sheep anti-human TF Ab was performed separately to determine the origin of TF.

A. TF and CD106 expression (open) on PAECs (black: isotype control) by flow cytometry.

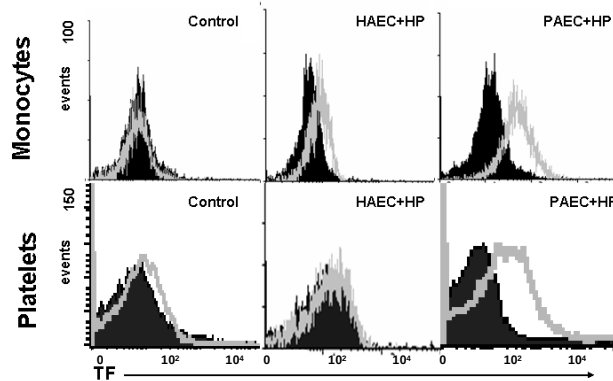
B. Recalcified clotting assay using normal plasma with (black) or without (open) pre-treated with sheep anti-human TF Ab after PAECs were pre-incubated in the presence (left) or absence (right) of fresh human plasma.

C. Recalcified clotting assay using FVII-deficient plasma after PAECs were pre-incubated in the presence (left) or absence (right) of fresh human plasma. (ns = no significant difference, HP = human plasma, HI-HP = HI human plasma, Mon = monocytes, PLT = platelets).

3.2.3 Procoagulant activity of human monocytes and platelets in the presence of human plasma

To determine the procoagulant effect on monocytes or platelets of 8-hour co-incubation with PAECs and human plasma, these cells were isolated and analyzed by flow cytometry. Low levels of TF expression were detected on monocytes after coincubation with HAECs and human plasma but not on platelets. However, TF expression was significantly induced on both monocytes and platelets after coincubation with PAECs in the presence of human plasma (Figure 3.2-3A). In clotting assays with recalcified normal plasma, clotting times induced by these cells were shortened compared to those induced by untreated monocytes or platelet controls (Figure 3.2-3B), but only in normal, not FVII-deficient plasma clotting assays (Figure 3.2-3B), demonstrating that this shortening was TF-dependent. The anti-human TF Ab inhibited shortening of the clotting time, consistent with the findings flowcytometry, indicating human TF was expressed on monocytes and platelets. Incubation with PAECs in the presence of human plasma therefore induced pro-coagulant human TF expression on both monocytes and platelets.

A



B

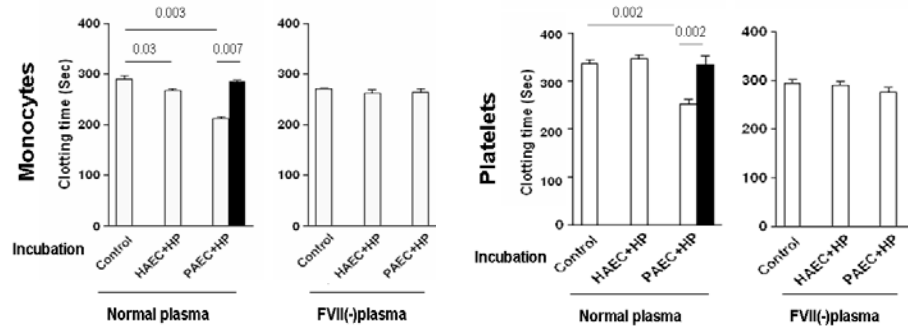


Figure 3.2-3 Human monocytes and platelets express human TF in the presence of human plasma.

Human monocytes and platelets were harvested from supernatants after co-incubation with HAECs or PAECs in the presence of 5% human plasma for 8 hours.

A. TF expression (open) on monocytes and platelets (black: isotype control) by flow cytometry.

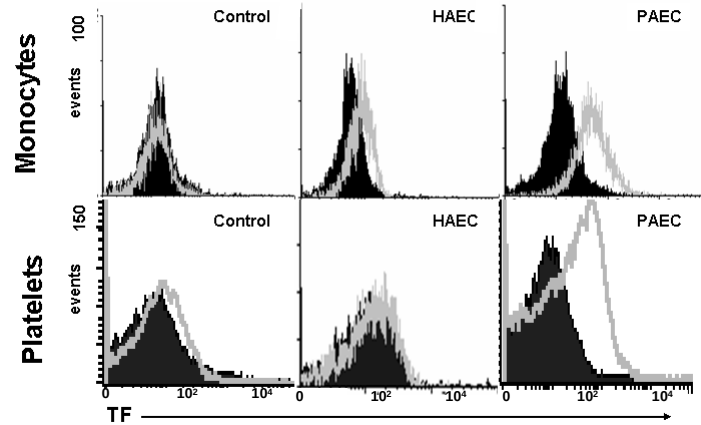
B. Clotting times of recalcified normal (left) and FVII-deficient (FVII(-)) (right) clotting assays in the presence of resting (control) or pre-incubated (with HAECs+HP or PAECs+HP) human monocytes or platelets. Clotting assays were also performed

following prior incubation of the monocytes or platelets with sheep anti-human TF Ab (black)

3.2.4 Procoagulant activity of human monocytes and platelets in the absence of human plasma

To determine the contribution of human plasma to the induction of TF, these experiments were repeated without human plasma in the incubation step, so that monocytes and platelets were incubated with PAECs alone. Under these conditions, TF was still induced on both monocytes and platelets (Figure 3.2-4A). In clotting assays, the results using monocytes were identical to those in the presence of human plasma, indicating that procoagulant human TF was induced on monocytes by incubation with PAECs (Figure 3.2-4B). Nevertheless, the changes in clotting times induced by activated platelets were different. Although the results from FVII-deficient plasma confirmed that shortened clotting times were TF-dependent, TF activity was not inhibited by anti-human TF Ab, indicating that procoagulant porcine TF was associated with platelets following incubation with PAECs.

A



B

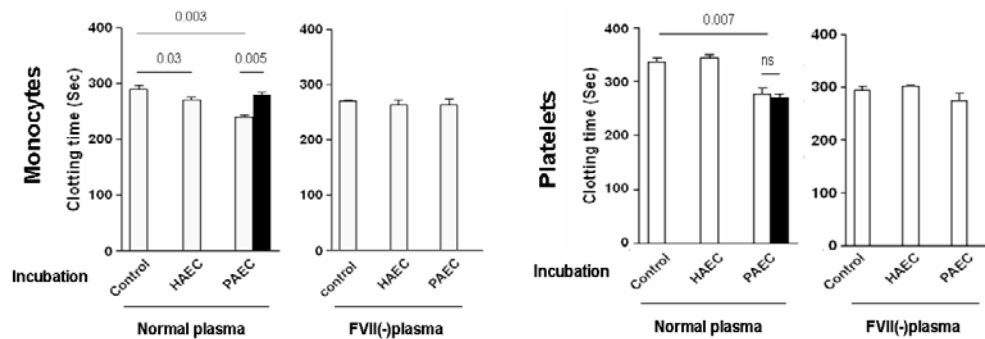


Figure 3.2-4 Human monocytes express human TF activity but platelets confer porcine TF activity in the absence of human plasma.

Monocytes and platelets were co-incubated with PAECs in the absence of 5% human plasma.

A. TF expression on monocytes and platelets (open) (black: isotype control) by flow cytometry.

B. clotting times of recalcified normal (left) and factor VII-deficient (FVII(-)) (right) plasma clotting assays involved with resting (control) or pre-incubated (with HAECs or PAECs) human monocytes or platelets. Coagulation assays were also performed

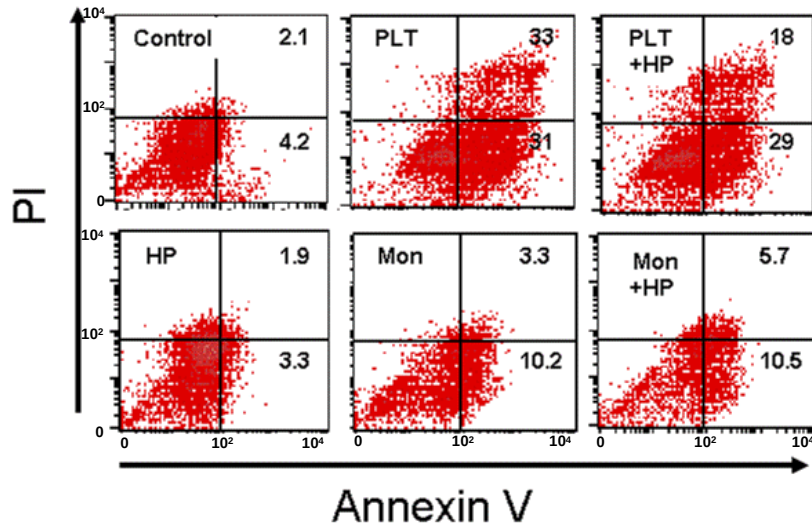
separately following prior incubation of activated the monocytes or platelets with the sheep anti-human TF Ab (black). (ns = no significant difference).

3.2.5 Human platelets induce PAECs apoptosis and shedding of TF-positive platelet-PAEC aggregates

To further investigate these results, we examined the interaction between human platelets and PAECs. After incubation with human platelets for 8 hours, the PAECs underwent significant apoptosis, as assessed by flow cytometry using annexin V and PI, which was partially inhibited in the presence of human plasma. In contrast, incubation with human monocytes provoked only a minimal increase in apoptosis of the PAECs (Figure 3.2-5A). Moreover, platelets were isolated after incubation with PAECs in the absence of human plasma, and stained with CD106 (red) and TF (green), and examined using immunocytochemical techniques (Figure 3.2-5B). Compared to control platelets, platelets incubated with PAECs became TF-positive, CD106-negative (arrow), indicating that the encounter with PAECs had activated them. However, this analysis clearly revealed the presence of larger, irregular particles that were double-positive for both markers (arrowheads). These were assumed to be fragments of apoptotic PAECs that expressed porcine TF and attached with platelets. The presence of significant quantities of these PAEC-platelet aggregates in the purified platelets accounted for the clotting time results (shown in Figure 3.2-4B), suggesting the presence of porcine TF.

A.

Porcine EC



B.

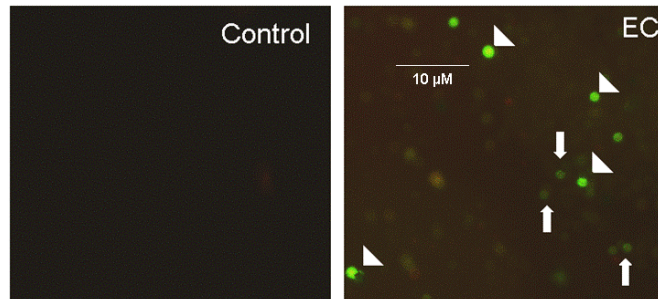


Figure 3.2-5 Human platelets induce PAECs apoptosis.

A. PAECs were isolated after co-incubation with various stimuli for 8 hours. Apoptosis was measured by flow cytometry. The percentage of cells is indicated.

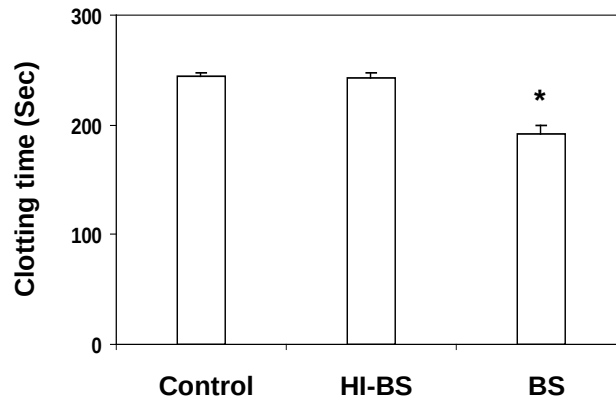
B. Immunocytochemical analysis of platelets following co-incubation with or without PAECs for 8 hours. Cells were stained with the sheep anti-human-TF Ab (FITC, green) and anti-porcine CD106 Ab (TRITC, red), then examined by fluorescence microscopy. TF-positive and CD106-negative cells are indicated by arrows and assumed to be

activated platelets. The larger, irregular particles double-positive for both markers are indicated by arrowheads; these were assumed to be fragments of apoptotic PAEC, which expressed porcine TF and attached to activated platelets (platelet-PAEC aggregates).

3.2.6 Procoagulant activity of PAECs, baboon platelets and PBMCs

Since baboons are a commonly-used primate surrogate for humans in pig-to-primate xenotransplantation models, all experiments were repeated by incubating PAECs with baboon platelets and PBMCs in the presence or absence of baboon serum. Monocytes were replaced by PBMCs because the Abs for selecting baboon monocytes were not available. The results of clotting assay were identical to those found in experiments with human components. PAECs were activated to express TF only in the presence of complement, and PAECs activated baboon platelets and PBMCs to express TF activity independent of the presence of baboon serum (Figure 3.2-6).

A



B

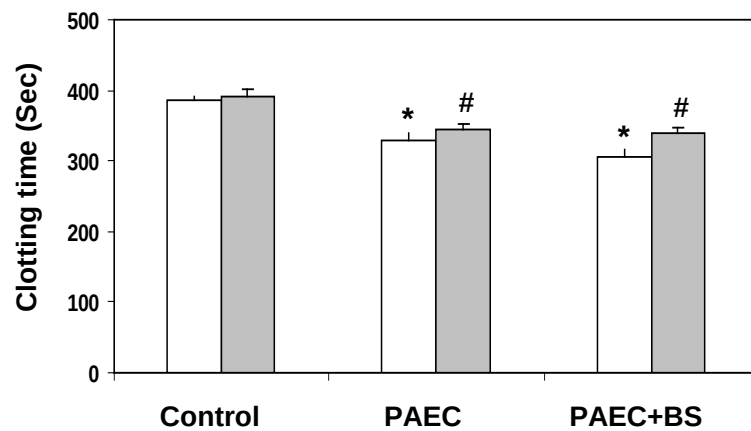


Figure 3.2-6 Baboon serum activates PAECs and PAECs activate baboon platelets and PBMCs to TF activity.

- A. PAECs were incubated with medium (control), fresh or HI baboon serum (BS) for 8 hours. TF activity of PAECs was determined by clotting assay in normal plasma.
- B. Baboon platelets and PBMCs were incubated with medium (control) or PAECs with/without BS for 8 hours. TF activity of PBMCs (white) or platelets (grey) was determined by clotting assay in normal plasma. (*, #: $p < 0.05$, compared to control group respectively)

3.3 DISCUSSION

The expression of TF has been increasingly recognized during HAR or AHXR (Gollackner et al., 2004; Ierino et al., 1998; Nagayasu et al., 2000) and linked to the activation of PAECs. The factors resulting in procoagulation have been hypothesized to play an important role in early xenograft failure. Most previous studies have focused on EC activation by XNA or/and subsequent complement activation as the trigger to coagulopathy and graft failure after xenotransplantation. However, two other factors are important to consider.

First, molecular incompatibilities of the coagulation-anticoagulation system of pig and primate may also contribute to the development of coagulation disorder. For example, porcine vWF aggravates the thrombotic process (Schulte Am Esch et al., 2005), and, conversely, TFPI and thrombomodulin fail to down-regulate the propagation of thrombosis (Kopp et al., 1998a; Kopp et al., 1998b; Siegel et al., 1997). Second, thrombosis can be initiated not only by TF activity on ECs but also by blood-borne TF, expressed by monocytes, platelets or circulating microparticles, all of which may have prothrombotic activity and substantially contribute to the formation of thrombosis (Bogdanov et al., 2003; Falati et al., 2002).

My study has attempted to address the relevance of this blood-borne TF as a potential mechanism that may contribute to the coagulopathy seen in AHXR. The present study confirmed that PAECs express TF after incubation with human plasma, and that this effect is lost after with HI human plasma, suggesting that it is a complement-dependent

process, compatible with the previous findings of other groups (Gollackner et al., 2004). We have assumed this is due to the presence of XNA, so it could be labeled as a procoagulant effect that is humoral immune response-dependent.

These results also clearly show that resting platelets and monocytes are unable to up-regulate TF expression of PAECs, consistent with other studies that have shown that only activated platelets can activate PAECs to express TF (Bustos et al., 2001; Xu et al., 2001). In an allogeneic setting, one study has shown that activated, but not resting monocytes were able to activate human ECs in allogeneic experiments. Although others reported resting monocytes could also upregulate TF expression on human ECs (Fan et al., 1993).

In systemic inflammatory processes monocytes and platelets are activated to express TF. For example, it has been previously reported that monocytes can be activated by allogeneic or xenogeneic EC (Kopp et al., 1998b). These results also revealed that monocytes were weakly activated by HAECs but much stronger by PAECs. However, there are no reports of platelets expressing TF in AHXR. In this study, resting PAECs activated monocytes and platelets to express TF, without the requirement of human plasma. This is clearly independent of the action of XNA, so could be labeled as being humoral immune response-independent. The situation appears complex for platelets, but mainly because, in the absence of human plasma, PAECs undergo significant apoptosis, and contribute to the formation of platelet-PAEC aggregates. These porcine TF-positive aggregates then resulted in the enhanced clotting seen in re-calcified clotting assay. Nevertheless, the staining experiments showed that platelets are activated in these

circumstances to express human TF, so we can state with some confidence that PAECs induce TF expression on platelets independently of XNA, and therefore independently of a humoral immune response.

These results suggest that recipient monocytes and platelets may play an important role in thrombotic complications during porcine organ xenotransplantation, and that overcoming this complication will not be achieved solely by further manipulation of the humoral immune response against the xenograft.

An important question is whether the apoptosis on PAECs after incubation with human platelets and resulting shedding of TF-positive aggregates that we observed is relevant to thrombotic complication. Although AHXR is mainly associated with ECs activation and fibrin deposition along the wall of blood vessels, rather than apoptosis in the endothelium (Holzknecht et al., 2002), in an animal study by transplanting human DAF transgenic kidney xenografts into baboons, numerous TUNEL-positive cells were detected with the destruction of the glomerular structure in the grafts, concomitant with the development of TM (Shimizu et al., 2005). The present study showed both human platelets and monocytes, but not human plasma alone, can induce apoptosis of PAECs (by detection of annexin V expression). Notwithstanding, how platelets induced PAECs apoptosis remained further investigating in xenotransplantation, platelets has been shown to induce ECs apoptosis in sepsis model(Kuckleburg et al., 2008). The disruption of integrity of the ECs and exposure of phosphatidylserine provoke the ECs to become prothrombotic

(Bombeli et al., 1997), so that any degree of apoptosis of luminal ECs *in vivo* might have some implications to thrombosis.

Unlike humans with A, B and/or H antigens, only A and H antigen express on the epithelial cell in the genetically-defined porcine blood group system. Importantly, they are not present on vascular ECs (Oriol et al., 1993). Furthermore, only blood types A and O (most are blood type O) exist in pigs. In these experiments, the ABO antigen of the pig was not determined, but was presumably A or O (Busch et al., 2006). Therefore, blood type A human plasma, platelets and monocytes were used as the recipient blood microenvironment to minimize the side effects of ABO-incompatibility transplantation.

In summary, although there were some limitations of the current model such as no shear force in incubation or no *in vivo* experiments, which can be carried out in the absence of plasma, a novel *in vitro* model system was defined to examine independently the contribution and interaction to thrombosis/TM made by PAECs, human platelets and monocytes. I have demonstrated that each may contribute to the expression of procoagulant TF after pig-to-primate xenotransplantation. The model is expected to be useful to investigate the mechanism of TM after xenotransplantation and to provide insights into the type of graft manipulation that could modify the process.

CHAPTER 4

ANTI-NONGAL IgG ANTIBODIES

ACTIVATE PAECS TO EXPRESS TF

INDEPENDENT OF COMPLEMENT

4.1 INTRODUCTION

The expression and activity of TF plays a significant role during AHXR. *In vivo* expression of TF was upregulated in necrotic xenografts (Nagayasu et al., 2000; Shimizu et al., 2005). *In vitro*, TF on PAECs was activated by the binding of anti-pig Abs and complement activation (Gollackner et al., 2004). The study in chapter 3 indicated that TF activity on PAECs was increased only in the presence of complement, but not by Abs, monocytes, or platelets alone (Lin et al., 2008).

The generation of GT-KO pigs has increased interest in investigating the role of anti-nonGal Abs in coagulation in pig-to-primate xenotransplantation models. Despite the effect of anti-Gal Abs have been successfully eliminated by neutralizing or depleting Gal Abs, most graft were still rejected by AHXR within a few weeks. It has been speculated that these grafts were rejected because of the presence and induction of anti-nonGal Abs. Recently, anti-nonGal Abs were indeed elevated in DAF (+/+) pig-to-primate heart or kidney xenotransplantation models (Chen et al., 2006; Lam et al., 2004).

TFPI is the crucial regulator of coagulation pathway, which is initiated by TF. *In vitro*, expression of hTFPI-CD4 on immortalized PAECs effectively prevented pTF-dependent clotting induced by TNF- α (Chen et al., 1999b). In this study, we investigated the role of nonGal Abs in coagulation during xenotransplantation and also tested whether TFPI transgenic PAECs were resistant to activation of anti-nonGal Abs.

4.2 RESULTS

4.2.1 Naïve baboon serum activates PAECs to express TF dependent on complement

PAECs were incubated with fresh or HI naïve BS for 8 hours, WT PAECs were induced to express TF activity in the recalcified clotting assay only by fresh but not by HI naïve baboon serum (Figure 4.2-1A). This confirmed previous study that activation of PAECs is dependent on the presence of complement in chapter 3. To determine whether PAECs transgenic for hCRP are resistant to the activation of primate serum by regulating complement activity, MCP (+/+) PAECs were incubated with fresh naïve BS. Naïve BS failed to activate MCP (+/+) PAECs to express TF activity by recalcified clotting assay (Figure 4.2-1A). Functional TF activity was mirrored by the expression of RNA, which was measured by quantitative PCR after PAECs were pre-incubated with serum for 4 hours (Figure 4.2-1B).

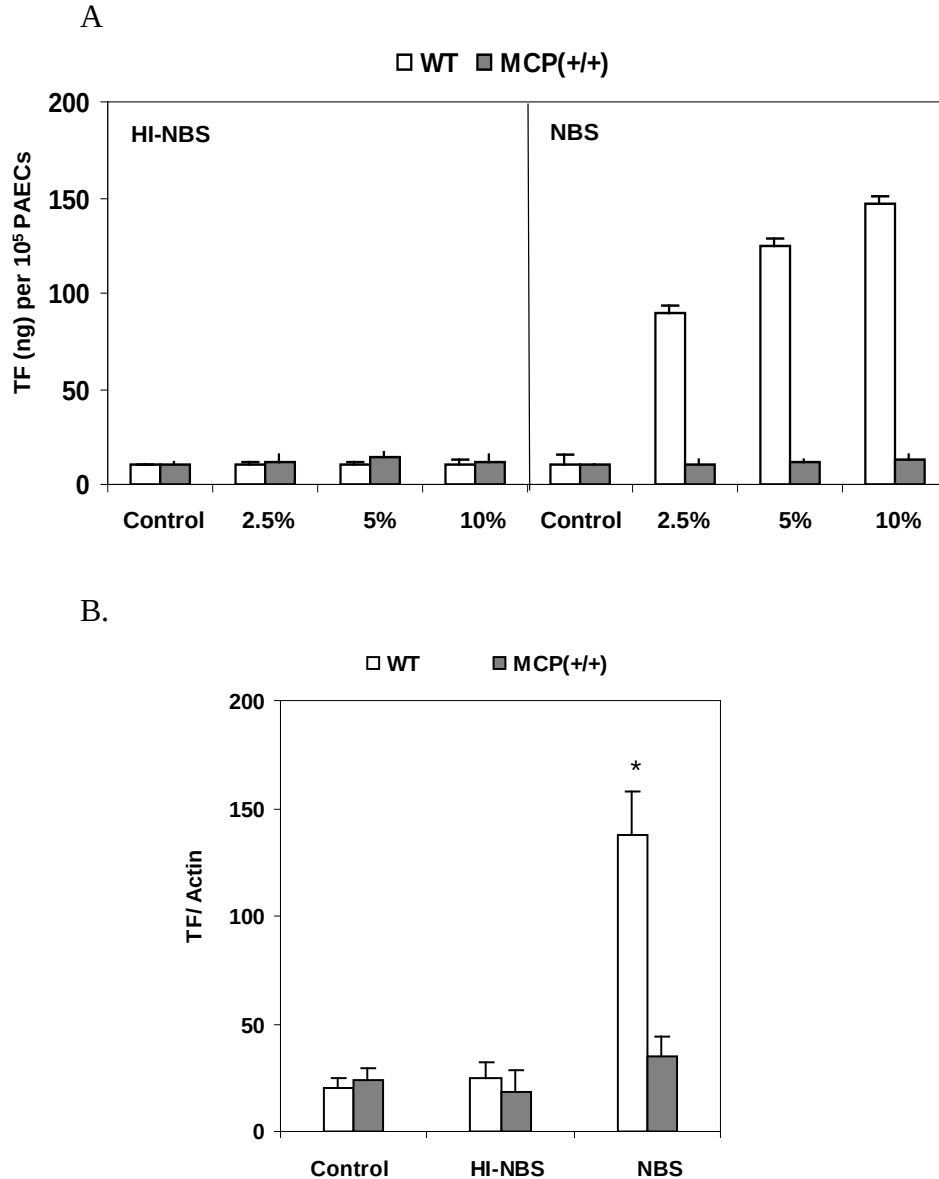


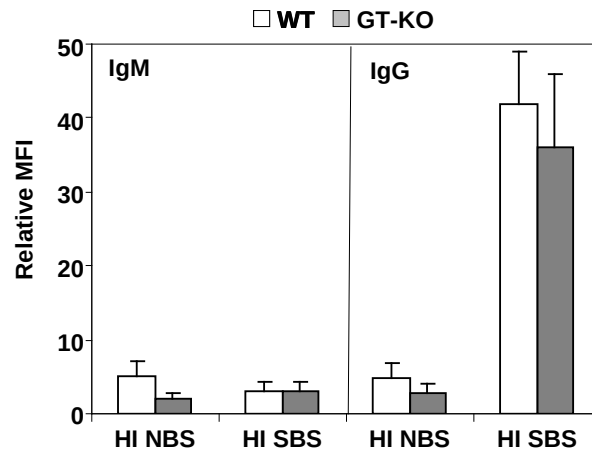
Figure 4.2-1 Naive baboon serum activated PAECs to express TF that is dependent on complement.

- A. WT or MCP (+/+) PAECs were incubated with medium (control) and a series of concentrations of fresh or HI naïve baboon serum (NBS) for 8 hours. The TF activity on the surface of PAECs was determined by the recalcified clotting assay.
- B. The experiments were repeated with incubation for HI-NBS for 4 hours. The TF expression of PAECs was determined by quantitative PCR (*p<0.05 vs control).

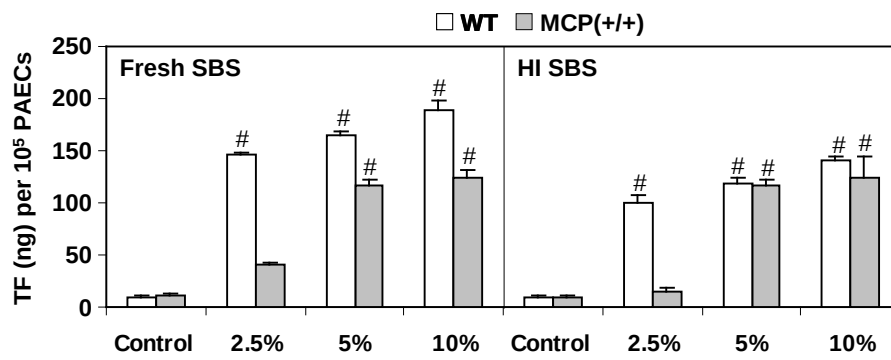
4.2.2 Sensitized baboon serum activates PAECs to express TF independent of complement

To determine the effect of anti-nonGal Abs on PAECs, sensitized baboon serum was collected from baboons that received aortic patch transplant from GT-KO pigs without immunosuppressants 4 weeks previously. Anti-nonGal Abs were determined by measuring the IgG and IgM binding of serum to GT-KO PAECs by flowcytometry. Sensitized baboon serum conferred 5 times higher titres of anti-nonGal Abs compared to naïve baboon serum (Figure 4.2-2A). The experiments were repeated by incubating PAECs with fresh and HI sensitized baboon serum. WT PAECs were activated to express TF activity by recalcified clotting assay not only by fresh sensitized but also by HI sensitized baboon serum (Figure 4.2-2B). Moreover, the MCP (+/+) PAECs were not resistant to the activation of fresh and HI sensitized baboon serum (Figure 4.2-2B). This indicated that sensitized baboon serum with high titres of anti-nonGal Abs was able to activate PAECs independent of the involvement of complement. This effect resulted from the presence of anti-nonGal Abs since HI sensitized but not naïve baboon serum activated GT-KO PAECs to express TF (Figure 4.2-2C). The TF functional activity by the recalcified clotting assay was compatible with the finding of RNA expression by quantitative PCR (Figure 4.2-2D).

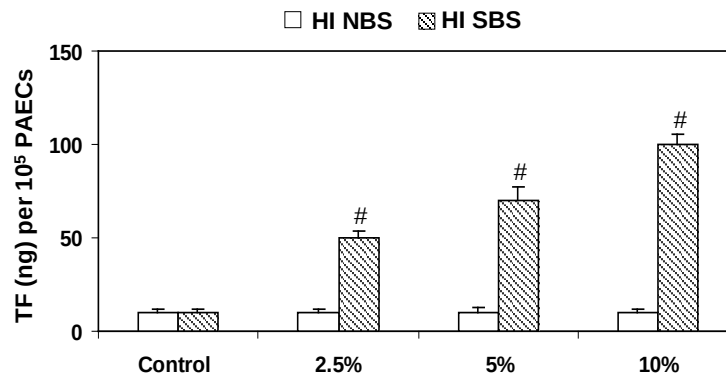
A



B.



C



D

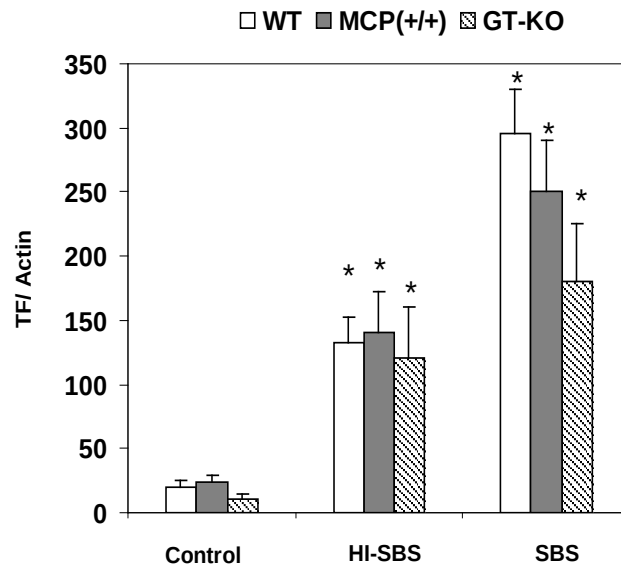


Figure 4.2-2 Sensitized baboon serum activates PAECs to express TF activity independent of complement.

- A. The IgG and IgM bindings of naïve (N=15) or sensitized baboon serum (N=3) to PAECs were determined by flowcytometry. The binding to GT-KO PAECs determines the binding of anti-nonGal Abs.
- B. WT or MCP (+/+) PAECs were incubated with medium (control) and a series of concentrations of fresh or HI sensitized baboon serum (SBS) for 8 hours. The TF activity on the surface of PAECs was determined by the recalcified clotting assay (#: $p < 0.01$ compared to control).
- C. GT-KO PAECs were incubated with medium (control) and HI naïve (NBS) or sensitized (SBS) baboon serum.
- D. WT, MCP (+/+), GT-KO PAECs were medium (control) and 10% of fresh or HI-SBS for 4 hours. The TF expression of PAECs was determined by quantitative PCR (* $p < 0.05$ vs control).

4.2.3 Sensitized baboon serum activates PAECs to express TF via IgG

To determine which anti-nonGal Abs activates PAECs to express TF activity, dithiothreitol (DTT) was used to destruct the disulfide bonds of IgM and eliminate the activity of IgM. The IgG binding of sensitized baboon serum to PAECs remained the same, but IgM binding disappeared after HI sensitized baboon serum was pre-treated with DTT for 30 minutes (Figure 4.2-3A). The HI sensitized BS, which was pre-treated with DTT activated WT PAECs to express the same level of TF activity, compared to non-treated serum (Figure 4.2-3B). This implied that the ability of anti-nonGal Abs to activate PAECs was unrelated to IgM and presumably related to IgG. To investigate further, HI sensitized baboon serum was pre-treated anti-human IgG Fab Ab for 30 minutes at 4 °C before incubation with PAECs. The anti-human IgG Fab Ab blocked the effect of HI sensitized baboon serum to induce WT PAECs to express TF activity (Figure 4.2-3C). This indicated that HI-sensitized baboon serum activated PAECs to express TF through IgG, but not IgM, Abs.

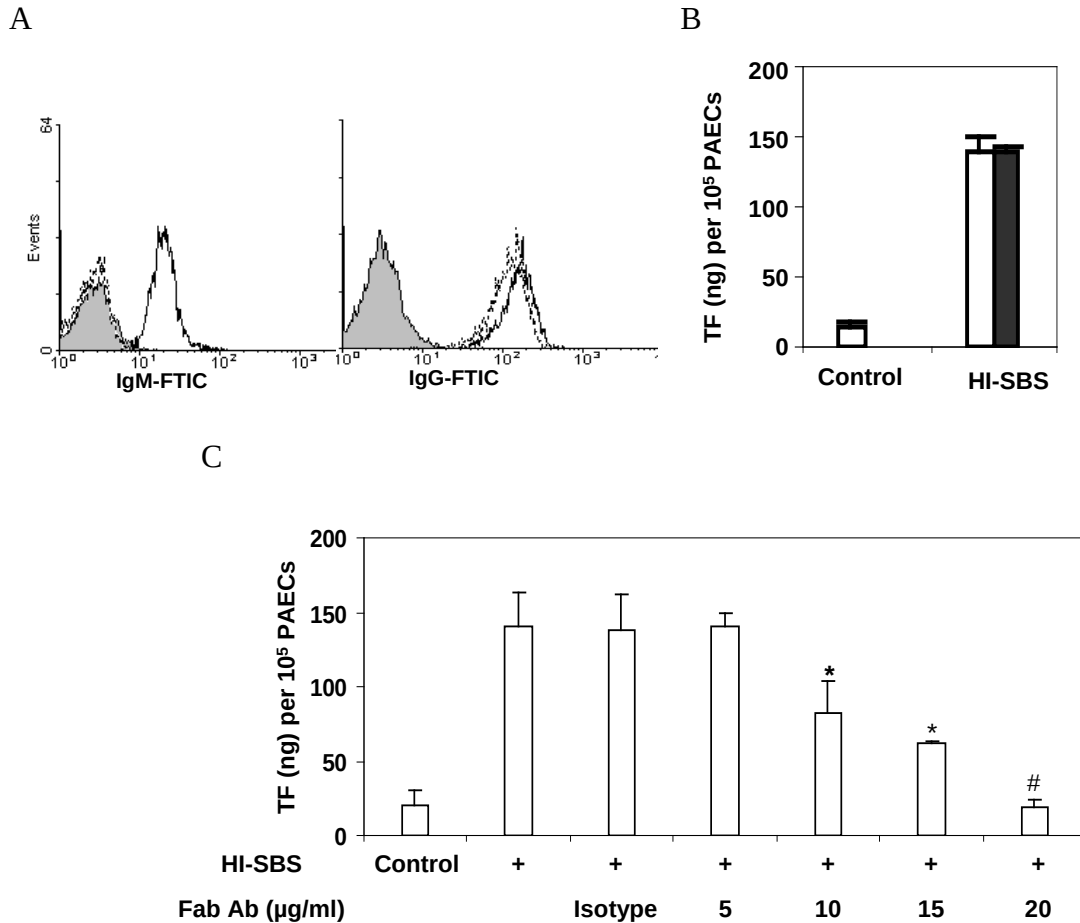


Figure 4.2-3 Anti-nonGal antibodies activate PAEC to express TF activity via IgG.

- A. IgG and IgM bindings of sensitized baboon serum (SBS) after pre-treated with dithiothreitol (DTT, 200 μ l/ml) to PAECs was determined by flowcytometry. Shadow: isotype, solid line: SBS, broken line: SBS treated with DTT.
- B. WT PAECs were incubated with medium (control) or HI SBS. The TF activity on the surface of PAECs was determined by the recalcified clotting assay. Black bar: HI SBS treated with DTT for 30 minutes.
- C. The same experiments were repeated with HI SBS, which had been treated with IgG Fab Ab for 30 minutes at 4 °C (* $p < 0.05$, # $p < 0.01$ vs untreated).

4.2.4 Human anti-nonGal natural antibodies activates PAECs to express TF

To determine the role of non-Gal Abs in human, human serum were collected from 15 volunteers. HI human sera were used to activate WT PAECs to express TF and anti-nonGal IgG Abs were also detected by flowcytometry. 13% (2/15) of sera had 5-fold high as titres of anti-nonGal Abs than other subjects. Consistently, the TF activity of PAECs induced by HI human serum was correlated with the titre of anti-nonGal Abs in human serum (Figure 4.2-4) HI human serum with high titre anti-nonGal Abs also activated MCP (+/+) PAEC to express TF activity (Figure 4.2-4B).

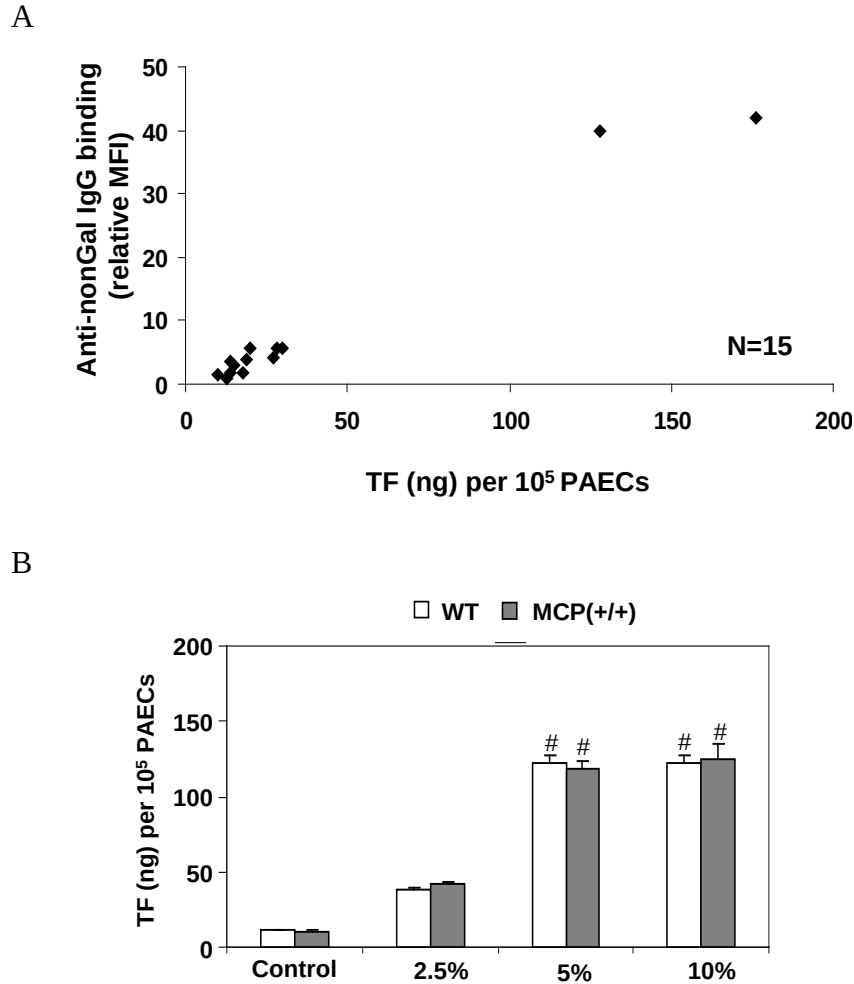


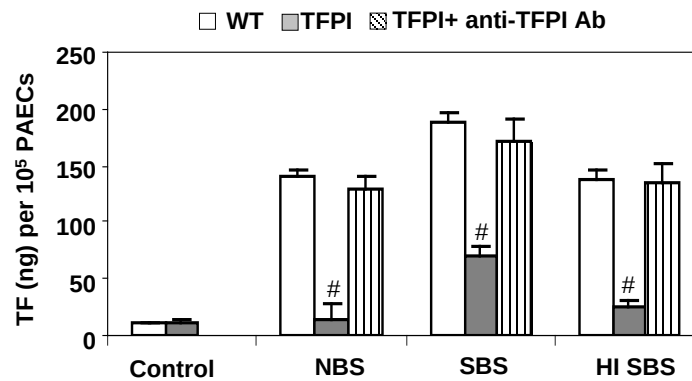
Figure 4.2-4 Human serum with high anti-nonGal antibodies activated PAECs to express TF independent of complement.

- A. The relationship between the titre of anti-nonGal IgG in human sera (n=15) and TF activity of PAECs, induced by HI human sera. The IgG binding of human sera to GT-KO PAECs was measured as anti-nonGal Abs. The same serum was used to activate PAECs and TF activity of PAECs, was determined by the recalcified clotting assay.
- B. MCP (+/+) PAECs were incubated with 10% fresh or HI human sera with high titre of anti-nonGal Abs (n=2). TF activity of PAECs was determined by the recalcified clotting assay (#: $P < 0.01$).

4.2.5 TFPI (+/+) PAECs inhibit the procoagulant effect of PAECs induced by baboon serum

The experiments were repeated by using TFPI (+/+) PAECs to clarify the effect of TFPI transgenic pigs. The TF activity was significantly attenuated in TFPI (+/+) PAECs, induced by fresh naïve baboon serum, fresh or HI sensitized baboon serum (Figure 4.2-5A). The inhibitory effect of TFPI (+/+) PAECs disappeared when anti-TFPI Ab was present (Figure 4.2-5A). However, RNA expression of TF on TFPI (+/+) PAECs still remained the same as those on WT PAECs after incubation with baboon serum (Figure 4.2-5B).

A



B

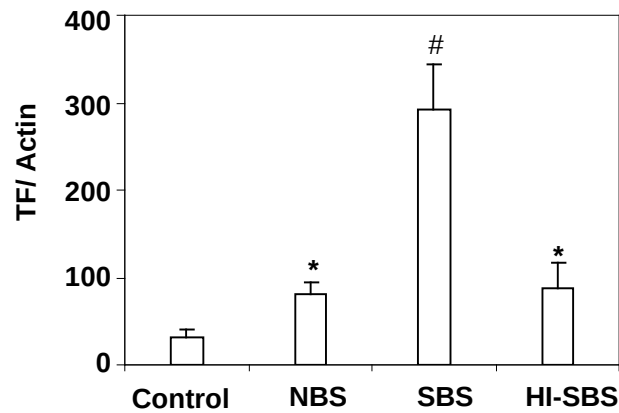


Figure 4.2-5 TFPI (+/+) PAECs inhibits TF activity on PAECs induced by naïve and sensitized baboon serum.

- A. TFPI (+/+) PAECs were incubated with medium (control) and 10% of naïve or sensitized BS for 8 hours. TF activity was determined by the recalcified clotting assay. (White bar: WT PAECs, grey bar: TPFI (+/+) PAECs, filled white bar: TPFI (+/+) PAECs were pretreated with anti-TFPI Ab (50µg/ml) for 30 minutes before the clotting assay) (#: $p < 0.01$, untreated vs treated with anti-TFPI Ab).
- B. TF expression was determined by quantitative PCR (* $p < 0.05$, # $p < 0.01$ vs control).

4.3 DISCUSSION

The expression of TF has been increasingly recognized during AHXR and linked to the activation of PAECs. Most previous studies have stressed that activation on EC as the trigger to thrombotic graft failure is dependent on the involvement of complement in response to XNA (Bach et al., 1994; Yang and Sykes, 2007). Abs alone failed to activate PAECs without the involvement of complement (Lin et al., 2008). Therefore, pigs expressing hCRP such as CD46 or CD55 are expected to protect ECs activation by regulating the complement activation. Our results showed that MCP (+/+) PAECs are resistant to the activation of naïve baboon serum even in the presence of complement. Previous report demonstrated that PAECs from GT-KO pigs additionally transgenic for CD46 were more resistant to primate complement-dependent cytotoxicity than those from GT-KO pigs (Hara et al., 2008).

The generation of GT-KO pigs removed the main XNA and initially was expected to attenuate the humoral immune response. However, anti-nonGal Abs emerges as an additional hurdle for successful xenotransplantation. For instance, anti-nonGal Abs were indeed elevated in DAF transgenic pig-to-primate heart or kidney xenotransplantation models (Chen et al., 2005; Chen et al., 2006). Moreover, recent study showed that after transplanting kidneys from GT-KO pigs to baboons, most of the baboons died from severe AHXR. Circulating induced Abs to nonGal antigens were markedly elevated at rejection, which mediated strong complement-dependent cytotoxicity against GT-KO porcine target cells (Chen et al., 2005).

In this study, we found that HI sensitized baboon serum with high titre of anti-nonGal Abs activated PAECs to express TF activity. In addition, PAECs that expressed MCP, which were resistant to the activation by naïve baboon serum with low titre of anti-nonGal Abs, was activated to express TF as well. We therefore concluded that anti-nonGal Abs activated a procoagulant phenotype on PAECs independent of the presence of complement. This activation was mainly derived from the effect of IgG Abs since it remained unchanged when IgM Abs were eliminated by the addition of DTT. These results confirmed the previous report that the elicited Abs, potentially to nonGal epitopes, induced PAECs activation and TF expression (Gollackner et al., 2004). Similarly, Saethre et al. reported human serum up-regulated E selectin expression on Gal depleted PAECs, which was largely complement-independent (Saethre et al., 2007). Importantly, anti-nonGal Abs do not only exist in sensitized baboons after transplantation; natural anti-nonGal Abs in over 10% of the population also had the same effect as elicited Abs if the titres were high enough. This implies that the organs from GT-KO pigs that express hCRP are not sufficient to overcome coagulopathy completely in pig-to-primate xenotransplantation model. This observation is mirrored in *in vivo* animal studies. Organ transplantation into nonhuman primates from GT-KO pigs that express a hCRP has been reported. Although the graft remained functioning, most recipients developed a consumptive coagulopathy, presumably associated with the procoagulant change on PAECs (Kelishadi et al., 2008).

In this study, we also demonstrated that primate serum up-regulated expression of mRNA of TF in PAECs by quantitative RT PCR. This can account, at least in part, for the

increased TF activity of PAECs after activation by primate serum seen by the functional recalcified clotting assay (Bach, 2006). Another explanation may be through the decryption of TF on the cell membrane. Procoagulant activity of TF is suppressed in the form of encryption in the cell surface. This function takes effect through decryption after stimulation and was evidenced to be associated with the function of TF on the surface of many cells (Rehemtulla et al., 1991; Bach, 2006; Pendurthi et al., 2007). Whether primate serum activates PAECs to express TF activity by decryption requires further investigation.

Since anti-nonGal Abs play an important role in ECs activation, interest has been directed to identify the anti-nonGal Abs. However, it is a formidable task to identify significant nonGal antigens, and then proceed to genetically modify pigs by deleting the gene for the antigen. A more reasonable approach is to generate GT-KO pigs that express anti-coagulant genes, such as TFPI, to prevent the development of a procoagulant phenotype when PAECs are activated, regardless of the presence of nonGal antigens. In a rodent model, Chen et al. reported that hearts from transgenic mice expressing membrane-tethered fusion proteins based on TFPI were transplanted into rats. In contrast to control non-transgenic mouse hearts, which were all rejected within 3 days, 100% of the organs from transgenic mice were completely resistant to humoral rejection and survived for more than 100 days when T-cell-mediated rejection was inhibited (Chen et al., 2004b). These data showed TFPI (+/+) PAECs can functionally inhibit TF activity on PAECs, activated by fresh naïve and HI sensitized baboon serum even though mRNA of TF is increased after stimulation. These promising results suggest transgenic anticoagulant genes, such as TFPI, will be beneficial to overcome thrombotic problems occurring in

AHXR. Actually, our group is waiting for the breeding of GT-KO pigs that express TFPI and MCP.

Anti-nonGal IgG activated PAECs to express TF activity through complement-independent pathway. This implies that GT-KO pigs expressing hCRP alone may be insufficient to prevent the development of procoagulant changes in PAECs. The effect can be inhibited by PAECs expressing TFPI. These results offer further approaches to prevent the development of coagulopathy during xenotransplantation.

CHAPTER 5

MOLECULAR INTERACTION BETWEEN

PRIMATE PLATELETS AND PORCINE

ENDOTHELIAL CELLS

5.1 INTRODUCTION

The activation and sequestration of platelets, leading to vascular thrombosis, is associated with HAR and AHXR. Once activated during xenograft rejection, platelets trigger the coagulation cascade in several ways. Activated platelets release endogenous coagulation factors to facilitate the coagulation cascade. The release of ADP and the receptor of GPIIbIIIa exert a positive feedback pathway to boost the activation of platelets. P-selectin on platelets or ECs upregulates the expression of TF on monocytes and mediates the binding of ECs with monocytes and neutrophils. The effect of P-selectin on monocytes in an area of vascular injury may be a component of a mechanism that initiates thrombosis (Celi et al., 1994). Moreover, platelet membrane aminophospholipids are exposed as a result of microparticle formation, which enhances the binding and assembly of the prothrombinase complex.

The mechanisms of platelet activation during xenotransplantation still remain elusive. As previously mentioned, these processes could be a consequence of the immunological response to the xenograft and viewed as a component of rejection. For example, the expression of vWF on activated ECs in response to XNA and complement and/or the generation of thrombin due to the activation of ECs are a potent stimulator of platelets. Furthermore, vascular injury could be further exacerbated by the associated loss of vascular ATPDase/CD39. The insufficient elimination of adenosine nucleotides may exacerbate vascular inflammation. Platelets binding to the C1q component of activated complement can result in the activation of the platelet fibrinogen receptor (GPIIbIIIa) with consequent expression of P-selectin, and development of procoagulant activity on

platelets(Peerschke et al., 1993). Nevertheless, non-immunologic factors, such as molecular incompatibilities, could also contribute to the activation of platelets and exacerbation of coagulation. Such molecular barriers could persist even if the xenogeneic immune response was completely inhibited.

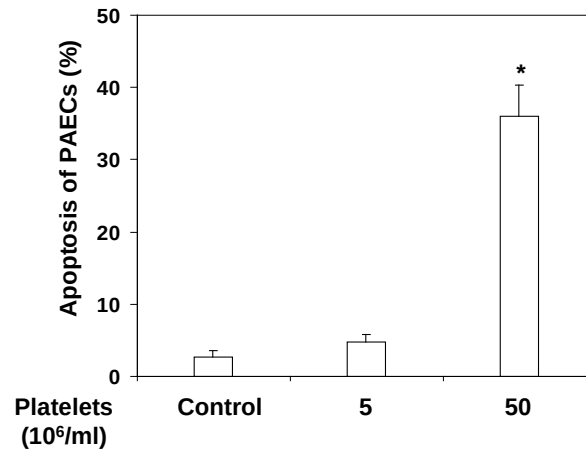
The results in Chapter 3 showed that PAECs could activate platelets and monocytes to express TF activity without the requirement of Abs and complement and therefore independent of the humoral immune response. Understanding the interactions between these cells would be helpful in developing systemic pharmacological approaches, such as anti-platelet modalities. In this chapter, we attempt to dissect the molecular interactions between primate platelets and PAECs.

5.2 RESULTS

5.2.1 Apoptosis of PAECs induced by platelets is associated with the quantity of platelets

In order to dissect the interaction between PAECs and platelets, the *in vitro* culture system was optimized to avoid the apoptosis of PAECs during incubation without the presence of primate (baboon or human) plasma shown in Chapter 3 (Figure 3.2-5). The extent of apoptosis of PAECs induced by incubation with platelets was associated with the concentration of platelets (Figure 5.2-1A). In this culture system, when PAECs were incubated with 5×10^6 /ml of (baboon or human) platelets, which was reduced from the initial 5×10^7 /ml of platelets, the apoptosis of PAECs decreased to the same percentage as when PAECs were incubated with medium. At the same time, PAECs activated platelets to express human rather than porcine TF since anti-human TF Ab inhibited TF activity expressed on platelets (Figure 5.2-1). Furthermore, the recalcified clotting assay in FVII-deficient plasma was optimized to be carried out with 3×10^6 /ml of platelets instead of 1×10^7 /ml to minimize the demand of platelets. In the following study of this chapter, all *in vitro* experiments were at least 3 times performed by using human and baboon platelets respectively.

A



B

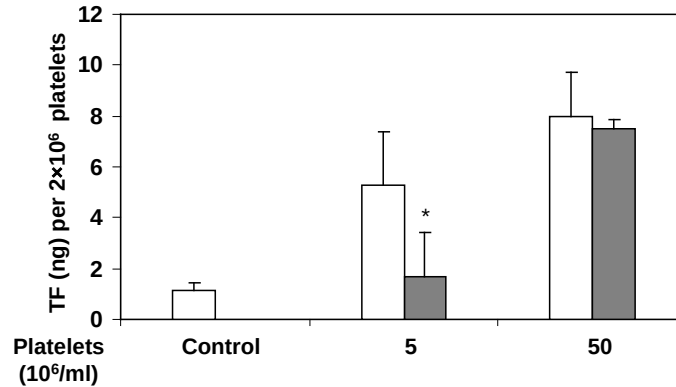


Figure 5.2-1 PAECs activate primate platelets to express primate TF.

A. PAECs were isolated after co-incubation with various concentrations of platelets (incubated with medium as control). Apoptosis was measured by flow cytometry, double-staining with annexin V-FTIC and PI-PE defined apoptotic cells. The percentages of cells double-positive for TF and annexin V or single-positive for annexin V are indicated. (*, $P < 0.05$)

B. Platelets were incubated with PAECs in the absence of human plasma for 8 hours. Platelets were harvested for analysis of TF activity by the recalcified clotting assay.

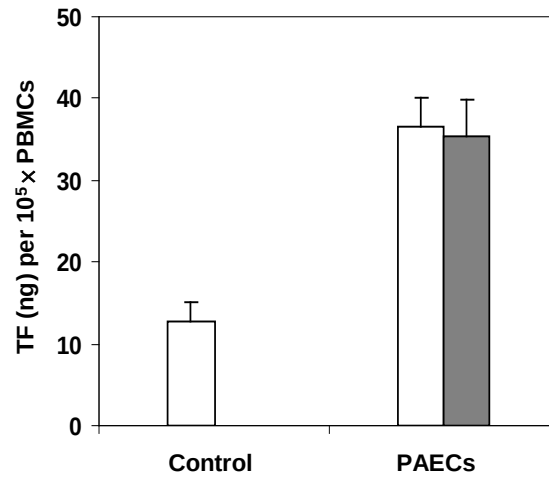
Platelets were incubated with anti-human TF Ab for 30 minutes prior to clotting assay (grey bar). (*, $P < 0.05$ compared to not pretreated with anti-TF Ab)

5.2.2 PAECs activates primate PBMCs and platelets to express TF activity

independent of cell-to-cell contact

To investigate the molecular interaction between PAECs and platelets, a transwell culture system (pore size: $0.4\mu\text{m}$) was used to determine the importance of cell contact. The experiments were carried out with the two cell types in a transwell system. The PAECs were cultured and adhesive in the lower chamber in all cases, and platelets or PBMCs were incubated in the upper chamber. In this system, PAECs still activated PBMCs and platelets to express TF activity, identical to the results in the non-transwell system (Figure 5.2-2).

A



B

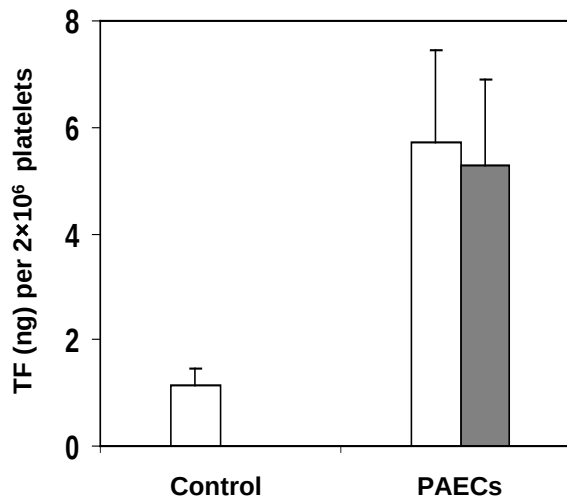


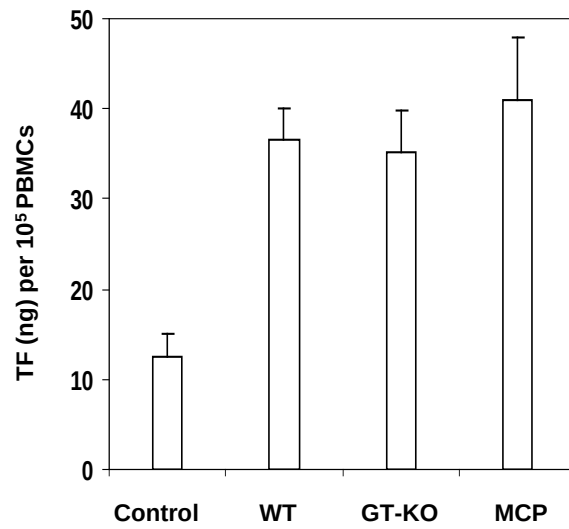
Figure 5.2-2 PAECs activate PBMCs and platelets to express TF independent of cell-to-cell contact.

PBMCs (A) and platelets (B) were incubated with medium (control) and PAECs in an ordinary and a transwell culture system for 8 hours. TF activity on PBMCs and platelets was determined by recalcified clotting assay (grey: transwell system).

5.2.3 TF activity on platelets and PBMCs is unrelated to the presence of the Gal antigen and the expression of a complement regulator

The generation of pigs that do not express Gal antigen or express a hCRP has overcome the challenge of HAR. To investigate lack of the expression of the Gal antigen or expression of a hCRP (CD46), PBMCs or platelets were incubated with PAECs from GT-KO pigs or MCP transgenic pigs. GT-KO PAECs and MCP (+/+) PAECs also activated primate PBMCs or platelets to express TF activity, identical to WT PAECs (Figure 5.2-4 A,B).

A



B

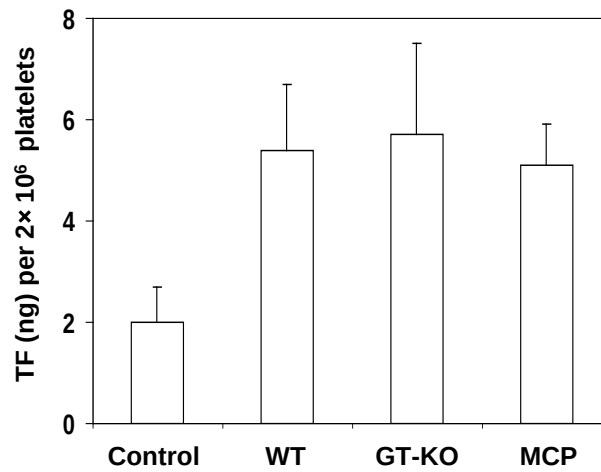


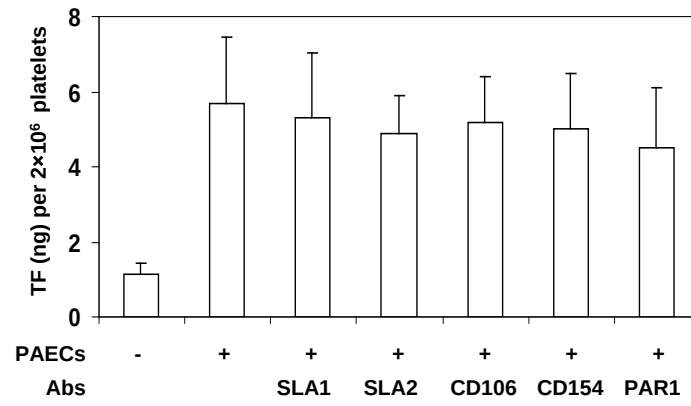
Figure 5.2-3 GT-KO or MCP (+/+) PAECs activate primate PBMCs and platelets to express TF activity identical to WT PAECs.

- A. PBMCs were incubated with medium (control) and various PAECs. TF activity on PBMC was determined by recalcified clotting assay.
- B. The same experiments were carried out with platelets.

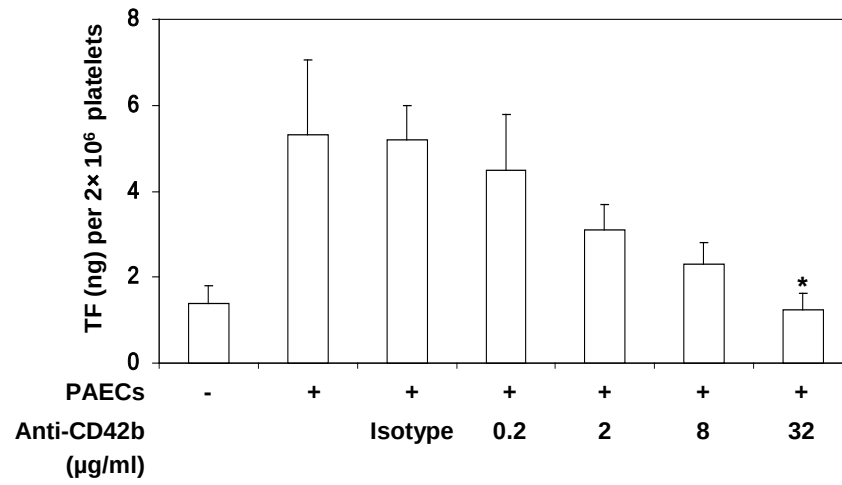
5.2.4 The molecular interaction between primate platelets and PAECs is associated with the receptor of GPIb α on platelets

We went on to analyze the molecular interaction between primate platelets and PAECs by an Ab-neutralizing method. The receptors and ligands possibly involved in this interaction were tested, including anti-SLA I, ant-SLA-II, anti-CD106, anti-CD42b, anti-P-selectin (clone 1.2B6), anti-CD154, and anti-PAR1 and anti-vWF Abs. TF activity on platelets was inhibited by the anti-CD42b (GPIb α), anti- vWF and anti- P selectin Abs, but not by other Abs (Figure 5.2-4).

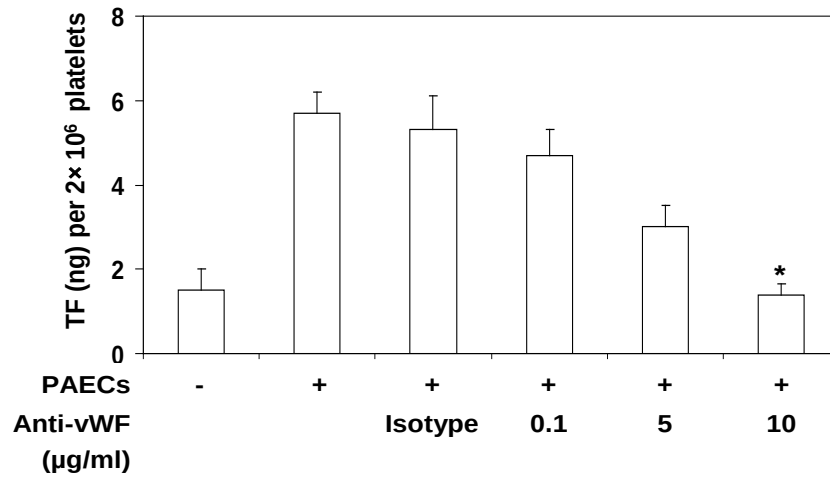
A



B



C



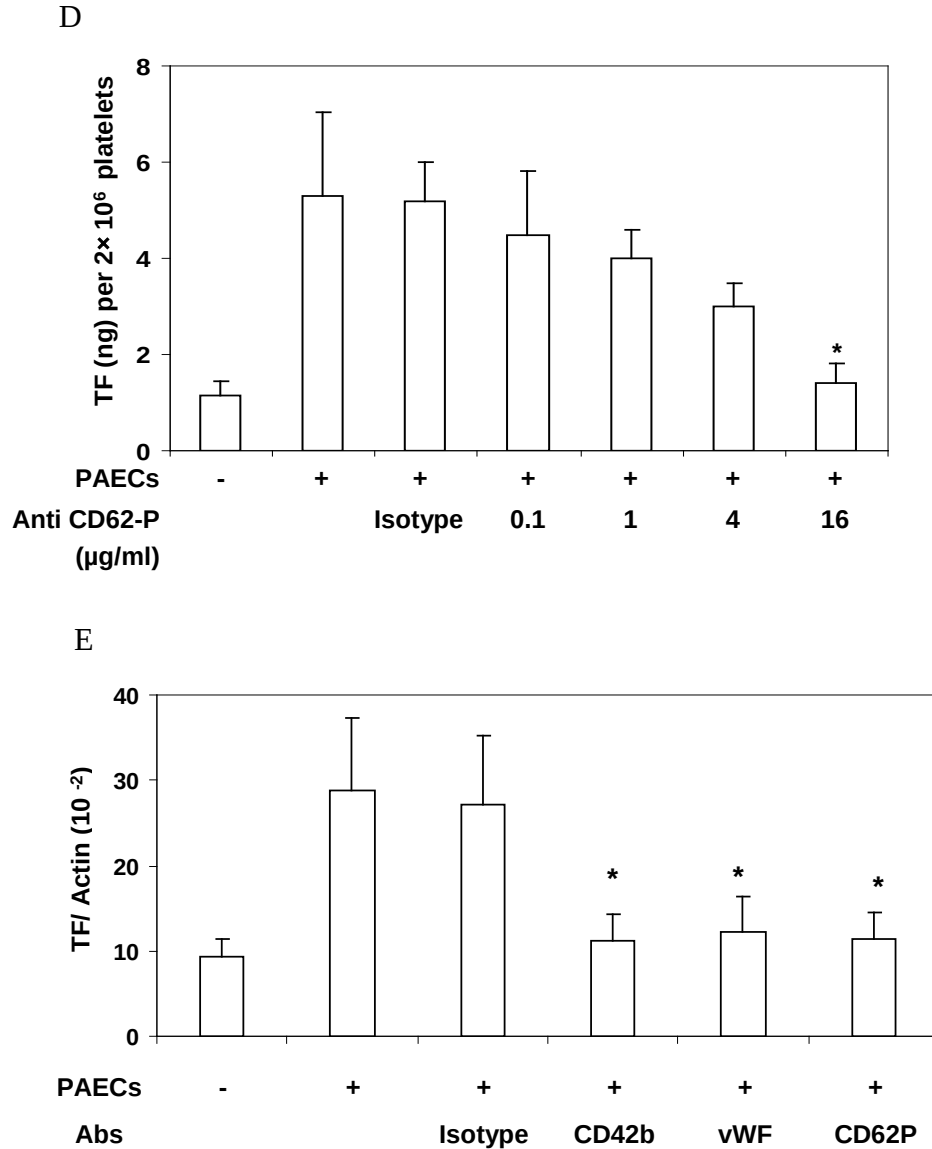


Figure 5.2-4 The interactions between PAECs and primate platelets are inhibited by anti-GPIb, vWF and P-selectin antibodies.

Both of primate platelets and PAECs were pre-treated with different neutralizing Abs for 30 minutes at 4°C. Thereafter, platelets were incubated with PAECs for 8 hours and harvested for analysis by the recalcified clotting assay (A, B, C, and D) and quantitative RT-PCR (E). (*, $P < 0.005$ compared to incubation with PAECs in each experiment. The concentration of isotype control was the same as the highest one of the Abs).

5.2.5 TF expression on primate platelets induced by PAECs is associated with the interaction between platelet P-selectin and PSGL-1

Since both platelets and ECs express P-selectin, we further investigated that the inhibitory site of anti-P-selectin Ab locates in primate platelets or PAECs. The experiments were performed by either washing or not washing Abs 3 times before primate platelets were added. Moreover, 3 clones of anti-P-selectin Abs were used and the reactivity to human or pig was determined by flowcytometry analysis (Figure 5.2-5) 1) Clone 1.2B6 is reactive to human P-selectin and human and pig E-selectin (Goda et al., 1999; Keelan et al., 1994; Wellicome et al., 1990). 2) Clone AK4 is reactive to human P-selectin. 3) Clone 12C5 is reactive to human and pig P-selectin (Stocker et al., 2000). TF expression on platelets after incubation with PAECs was inhibited by anti-human P-selectin Abs (1.2B6 and AK4) and anti-human and pig P-selectin Ab (12C5). After washing prior to incubation with primate platelets, anti-vWF Ab still inhibited TF expression on primate platelets but 3 clones of anti-P-selectin Abs did not (Figure 5.2-6). Moreover, anti-PSGL-1 Ab (CD162), the receptor of P-selectin on platelets, also prevented TF expression on platelets (Figure 5.2-7). These results indicated that anti-vWF Ab functioned on the surface of PAECs; in contrast, anti-P-selectin Abs acted on platelets.

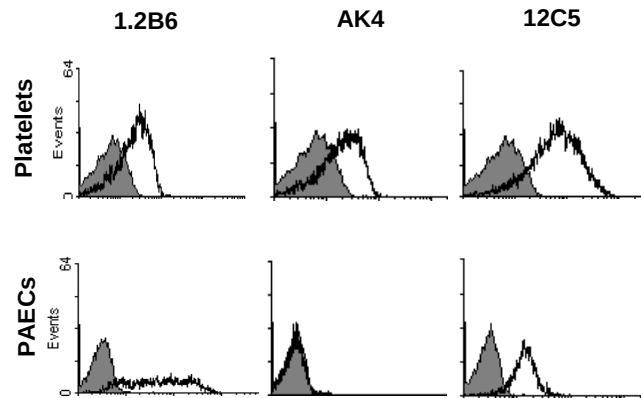


Figure 5.2-5 The reactivity to human or pig of 3 clones of anti-P-selectin antibodies.

Platelets and PAECs were activated by thrombin (1 U/ml) for 30 minutes or TNF- α (10ng/ml) for 2 hours respectively. The phenotypes were determined by flowcytometry. The clones of 1.2B6 and 12C5 are cross-reactive to both pig and primate but clone AK4 only is reactive to primate (Grey: isotype control).

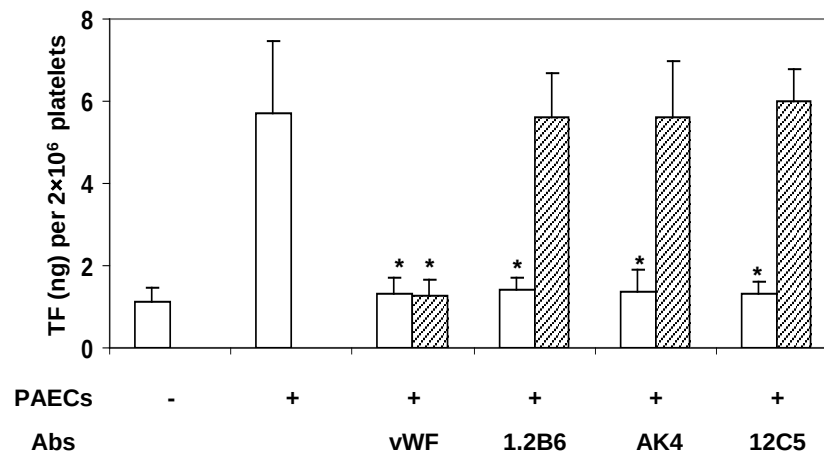


Figure 5.2-6 Anti-vWF and anti-P-selectin antibodies act on different sites to inhibit TF expression on primate platelets after incubation with PAECs.

PAECs were pre-treated with anti-vWF Ab or anti-P-selectin for 30 minutes; then primate platelets were co-incubated for another 8 hours. TF expression on platelets was analyzed

by recalcified clotting assay. (Filled: Abs were washed out before platelets were co-incubated, * $P < 0.05$ compared to without neutralizing Ab).

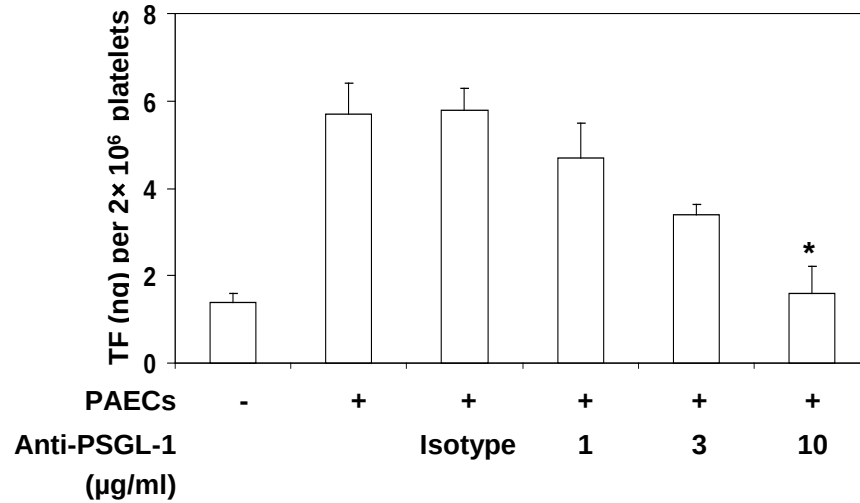


Figure 5.2-7 TF expression on platelets is inhibited by anti-PSGL-1 antibody.

Primate platelets and PAECs were pre-treated with different neutralizing Abs for 30 minutes at 4°C. TF activity on platelets was analysed by recalcified clotting assay. (*, $P < 0.005$ compared to incubation with PAECs. The concentration of isotope control was the same as the highest one of the Ab).

5.3 DISCUSSION

The origin of TF on platelets still remains controversial. Stimulated human platelets seem to express preformed TF which derives from the α -granules and the open canalicular system of resting platelets and is exposed on the cell surface after platelet activation (Muller et al., 2003). Platelet-leucocyte interactions seem necessary for its expression and TF is transferred to platelets by monocyte-derived microparticles (Morrissey, 2001). Recently, platelets have been demonstrated to synthesize *de novo* proteins via the splicing process of constitutive pre-mRNA (Denis et al., 2005). TF is also constitutively synthesized as well. This process is controlled by CLK1, and interruption of CLK1 signaling prevents TF from accumulating in activated platelets. In the present study, the TF activity of platelets after incubation with PAECs was dose-dependently inhibited by anti-CLK1 Ab. This result suggests TF expression on activated platelets is *de novo* synthesized and via splicing of pre-mRNA.

The interesting question regarding the interactions between primate platelets and PAECs is to determine the molecular interactions, by which we can target and develop new pharmaceutical approaches. In this present study, the data show that platelets were activated by PAECs to express TF activity without the requirement of cell-to-cell contact. The Ab neutralizing study indicates TF expression on platelets is inhibited by anti-GPIb (CD42b) and anti-vWF Abs. Although both PAECs and primate platelets express vWF, TF expression on platelets was not only inhibited in the presence of anti-vWF Ab, but also when Ab was washed out before platelets were co-incubated. Therefore, these observations suggest that PAECs secrete soluble vWF to activate primate platelets to

express TF via GPIb receptor on platelets. This result is compatible with other group's previous findings (Schulte Am Esch et al., 2005). Their studies indicated that purified porcine vWF spontaneously aggregates human platelets, which was inhibited by anti-human GPIb Ab. By transiently transfecting recombinant human or porcine vWF A1-domains in COS-7 cells, Schulte et al. reported that platelets were activated through aberrant interaction between the O-glycosylated A1 domain and platelet glycoprotein Ib in the absence of shear stress. This effect probably has *in vivo* significance, particularly after lung xenotransplantation. For instance, in an *ex vivo* pig-to-human pulmonary xenotransplantation model (Kim et al., 2008), pre-treatment of porcine lungs with desmopressin, which can reduce the content of vWF, attenuated platelet activation and systemic intravascular coagulation. Furthermore, if pulmonary intravascular macrophages were depleted in the recipients, baboons survived longer after receiving a lung transplant from a vWF-deficient pig than from a CD46-transgenic pig (Cantu et al., 2007).

P-selectin is an adhesion molecule expressed on activated platelets and ECs and plays an important role in atherosclerosis. P-selectin also circulates in plasma in a soluble form (sP-selectin), which induces procoagulant microparticle formation. However, that the thrombotic effects of P-selectin result from platelets or ECs still remains controversial. When monitored by intravital microscopy, platelets, like leucocytes, were found to roll on activated endothelium in the mesenteric venules and this process is supported by either vWF or P-selectin, dependent on shear rate (Andre et al., 2000; Frenette et al., 1995). Furthermore, GPIb was shown to be the counter-receptor not only for vWF, but for P-selectin (Andre et al., 2000). Therefore, P-selectin on ECs is inferred to play a more

important role in thrombosis. On the other hand, P-selectin of platelets was found to stabilize platelet aggregates (Merten and Thiagarajan, 2000). In an *in vivo* model of transluminal endothelial injury of the femoral artery, the platelet layer in P-selectin-deficient mice was less compact and did not recruit leucocytes as it did in WT mice (Smyth et al., 2001). These results indicated that P-selectin might support platelet–platelet or platelet-leucocyte interactions to promote fibrin deposition.

In this present study, TF expression on primate platelets induced by PAECs is inhibited by anti-human P-selectin Abs (clone 1.2B6, AK4 and 12C5). Contrary to anti-vWF Ab, which still inhibit TF expression on platelets when Abs are washed out before platelets are co-incubated, anti-porcine P-selectin Abs (clone 12C5) fail to inhibit TF expression on platelets. TF expression on platelets was inhibited by Ab for the receptor of P-selectin, PSGL-1. Hence, we conclude that this inhibitory effect of TF expression on platelets is attributed to P-selectin on platelet, but not on PAEC. Interestingly, in this system, the platelets were essential free of leucocytes, evidenced by flow cytometry, so the effect of anti-P-selectin Ab is irrelevant to the existence of leucocytes. Furthermore, a recent study indicates that platelets also have the receptor of P-selectin ligand (PSGL-1) (Frenette et al., 2000), the present study suggested that like ADP or GPIIbIIIa, expression of P-selectin on platelets may create positive feedback to activate platelets further via P-selectin and PSGL-1 pathway and contribute the activity of TF. This finding provides an alternative to inhibit TF expression in xenotransplantation by pharmaceutical inhibition of P-selectin ligand. On the other hand, what triggers resting PAECs to secrete vWF

emerges as an important entity to prevent TF expression on platelets and remains under investigation.

The generation of pigs that lack the expression of Gal epitopes reduces the immunologic response and increase interest in investigating the prevention of coagulopathy in xenotransplantation. During laminar flow, monocyte adhesion to PAECs is dependent on Gal-mediated monocyte activation (Peterson et al., 2005). Recruitment of human monocytes to non-activated xenogeneic ECs requires both α_4 and β_2 integrins on the monocyte; binding of Gal to monocytes results in rapid activation of β_2 integrins. Therefore, PAECs from GT-KO pigs would be expected to have a beneficial effect in preventing TF expression on monocytes. However, in the present study, PAECs that lack Gal epitopes did not have a protective effect and still activated primate PBMCs to express TF. Similarly, primate platelets were activated to express TF activity by GT-KO PAECs as well.

PBMCs are activated to express TF activity by PAECs through soluble molecule(s) in the present study. This finding is compatible with the early experiments. IL-1 mediated the increases of TF-associated procoagulant activity on ECs cocultured with allogeneic monocytes and stimulated with lipopolysaccharide and/or IgG (Wharram et al., 1991). Nevertheless, adhesion interactions in the induction of TF on monocytes which involves ligation with both E-selectin (Lo et al., 1995) and P-selectin (Celi et al., 1994) have been recognized.

Genetic deletion of Gal epitopes and the expression of a hCRP failed to attenuate the TF expression on platelets, which are activated by PAECs. These results were confirmed by *in vivo* studies (see Chapter 7). Platelet aggregation occurred early after xenotransplantation when the liver or kidney grafts from GT-KO/ MCP (+/+) pigs were transplanted into baboons. The severity of platelet aggregation was similar to those using WT organs. The identification of molecular interaction between primate platelets and PAECs as mentioned above is mandatory to facilitate further genetic manipulation or pharmaceutical approach.

CHAPTER 6

**PHARMACEUTICAL APPROACHES TO
PREVENT CONSUMPTIVE
COAGULOPATHY IN ACUTE HUMORAL
XENOGRAFT REJECTION: STATINS AND
ANTI-PLATELET AGENTS**

6.1 INTRODUCTION

In addition to genetic modification of organ donors, pharmaceutical intervention has been attempted to solve the procoagulant challenge. Interest is focused on platelet aggregation and thrombi because they may contribute to graft dysfunction by decreasing local blood flow. Activated platelets also promote leucocyte infiltration and localized vasoconstriction by the expression of P-selectin and secretion of ADP and serotonin. The history of using nonselective anti-platelet agents with differing mechanisms of action in experimental xenotransplantation dates back to as early as 1974 by Shons and Najarian, but with limited success(Shons and Najarian, 1974). In the final decade of the last century, therapy with more specific agents, such as platelet-activating factor (PAF) antagonists and anti-P-selectin Abs resulted in modest prolongation of survival of discordant xenografts (Coughlan et al., 1993; Makowka et al., 1990).

Statins are widely used to lower blood cholesterol levels and prevent ischemic heart disease. Clinical trials have demonstrated statins have pleiotropic effects, such as anti-inflammatory, anticoagulant, and immunomodulatory effects (Mason, 2003). Observations in heart transplantation patients, together with several *in vitro* studies that have determined a potential mechanism for their immunosuppressive effect, have provided a principle for the use of statins in patients with organ allografts (Kobashigawa, 2004).

Although the exact reasons of xenograft failure in pig-to-primate xenotransplantation remain a mystery, depending on such factors as the type of source-pig (wild-type or

genetically modified), the immunosuppressive regimen, and the nature of the transplanted organ or cells, they are indispensably associated with the interactions of the immune response, inflammation and coagulation. The pleiotropic effects of statins provide a promising opportunity to attenuate all the detrimental reactions at the same time. My colleagues have demonstrated that statin reduced in a dose-dependent manner the upregulation of swine leucocyte antigen (SLA) class II on PAECs, induced by IFN- γ (Ezzelarab and Cooper, 2007). Using mixed lymphocyte reaction, atorvastatin reduced the human PBMC proliferative response to unstimulated or stimulated PAECs from WT or GT-KO pigs (Ezzelarab et al., 2008). In this chapter, we investigated the anticoagulant and anti-inflammatory benefits of atorvastatin in xenotransplantation.

The constant feature of platelet microthrombi in AHXR also convinced us to re-examine the application of a novel anti-platelet drug as an adjuvant therapeutic agent. The integrin GPIIb/IIIa is crucial in the formation of platelet aggregates and potentiates adhesion to subendothelial matrices via fibrin(ogen), vWF, and vitronectin. Eptifibatide, a synthetic cyclic heptapeptide, is a selective high-affinity inhibitor of the platelet glycoprotein IIb/IIIa receptor. It has been demonstrated to be capable of inhibiting platelet-dependent prothrombinase activity in the acute coronary syndrome (Li et al., 2000). Another GPIIb/IIIa antagonist, tirofiban, also inhibits thrombin generation during cardiopulmonary bypass in baboons (Rao et al., 1999). ADP, which is secreted from ECs or activated platelets provides autocrine and paracrine function to augment the recruitment of platelets via the P2 receptor on platelets. More evidence showed that the ADP pathway is associated with the initiation and generation of thrombin. In P2Y1-deficient mice, the

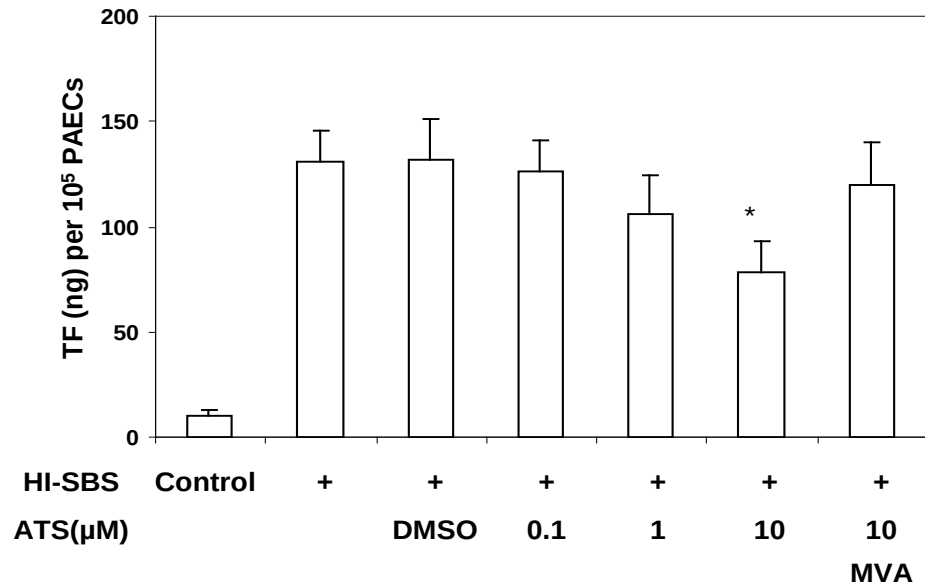
ability of collagen to enhance the generation of thrombin was impaired (Leon et al., 2004). Herein, we also investigated the beneficial effect of anti-platelet agents on TF expression on primate platelets after incubation with PAECs.

6.2 RESULTS

6.2.1 Atorvastatin inhibits TF activity on PAECs induced by anti-nonGal antibodies

To investigate the anticoagulant effect of atorvastatin in xenotransplantation, WT PAECs were pre-treated with atorvastatin with or without mevalonic acid for 16 hours before being co-incubated with HI-sensitized baboon serum. Atorvastatin inhibited about 50% of TF activity of activated WT PAECs by the recalcified clotting assay. This effect was associated with the presence of atorvastation since the addition of mevalonic acid, which is a downstream product of HMG-CoA reductase, reversed the inhibitory effect of atorvastatin (Figure 6.2-1A). Consistently, atorvastatin also inhibited the RNA expression of TF in WT PAEC after incubation with HI-sensitized BS by quantitative RT-PCR (Figure 6.2-1B).

A



B

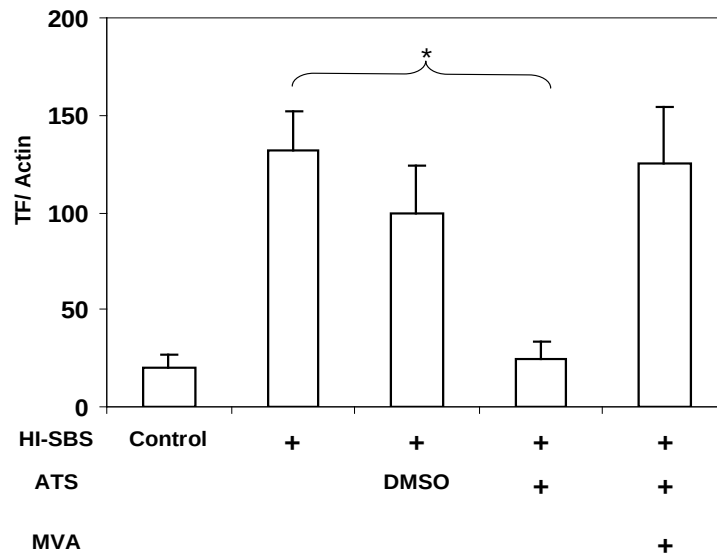


Figure 6.2-1 Atorvastatin inhibited TF expression on PAECs activated by heat-inactivated sensitized baboon serum.

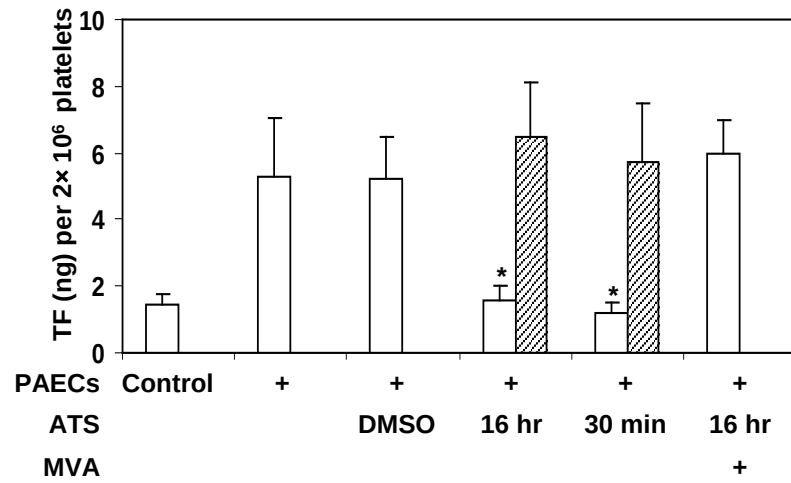
PAECs were incubated with various concentrations of atorvastatin without or with mevolonic acid (MVA) (10μM) for 16 hours. Atorvastatin was reconstituted in dimethyl sulfoxide (DMSO). Thereafter PAECs were activated by 10% HI-sensitized baboon

serum (SBS) for another 8 hours. TF activity on the surface of PAECs was determined by the recalcified clotting assays (A) and quantitative RT-PCR (B).

6.2.2 Atorvastatin inhibits TF expression on platelets activated by PAECs

We went on to investigate the ability of atorvastatin to inhibit the activation of platelets after incubation with PAECs. Platelets were pre-incubated with atorvastatin (5 μ M) for 30 minutes and 16 hours with/without mevalonic acid (10 μ M), and then were co-cultured with PAECs for 8 hours. TF activity was analyzed by the recalcified clotting assay. TF activity on platelets was inhibited after incubation with PAECs when platelets or PAECs were pretreated with atorvastatin both for 30 minutes and 16 hours (Figure 6.2-2A). However, the inhibitory effect disappeared if atorvastatin was washed out before primate platelets were added. The effect of atorvastatin was also negative when simultaneously incubated with mevalonic acid. The experiments were repeated with 2 hour incubation when PAECs was pretreated with atorvastatin for 16 hours for analysis of TF mRNA. The expression of mRNA was identical to the finding of the recalcified clotting assay (Figure 6.2-2B).

A



B

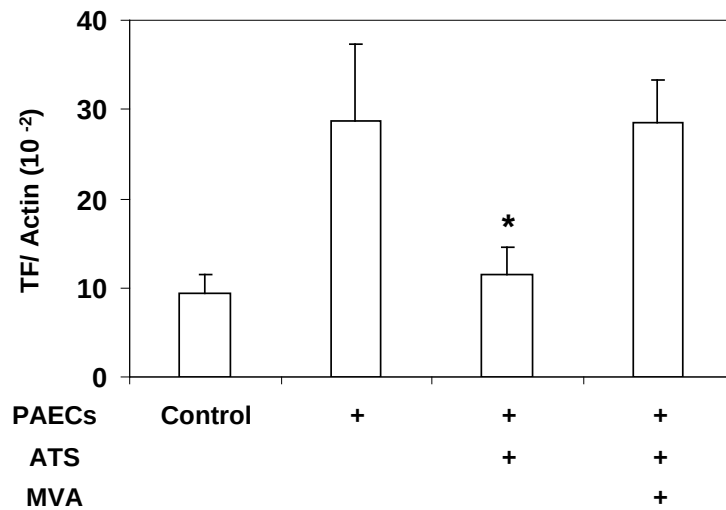


Figure 6.2-2 Atorvastatin inhibits TF expression on platelets after incubated with PAECs.

Primate platelets and PAECs were pre-treated with atorvastatin (ATS) for 16 hours or 30 minutes in the presence or absence of mevalonic acid (MVA) for 30min. (filled: atorvastatin was washed out before platelets were co-cultured.) Thereafter, platelets were

incubated with PAECs and harvested for analysis by the recalcified clotting assay (A) and quantitative RT-PCR (B). (*, $p < 0.05$ compared to incubation with PAECs alone).

6.2.3 PAECs induce the aggregation of platelets and PBMCs *in vitro*

The number of CD42a-positive cells of PBMCs was significantly higher 8 hours after incubation with PAECs than after incubation with HAECs or medium alone. After incubation for 24 hours, the percentage of CD42a-positive cells was slightly higher after incubation with HAECs than with medium, but was significantly increased after incubation with PAECs. Therefore, in further experiments to examine the effects of drugs, the experiments were carried out after incubation for 8 hours (Figure 6.2-3).

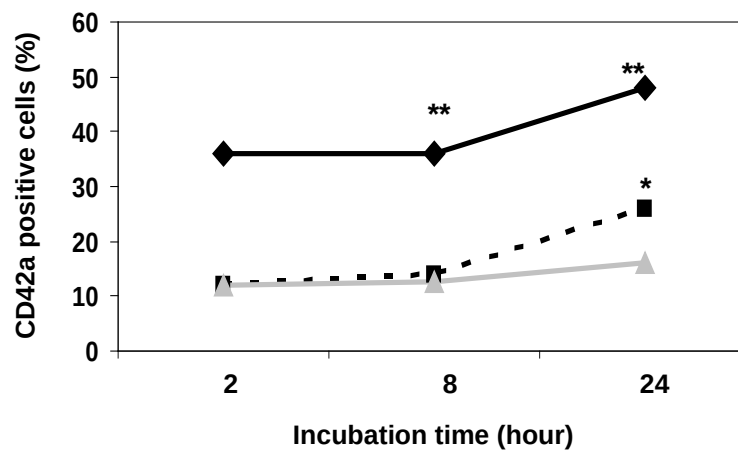


Figure 6.2-3 CD42a-positive PBMCs after incubation with platelets and PAECs.

PBMCs and platelets from the same donors were incubated with medium (control, grey line), HAEC (dash line), PAECs (solid line) for variable periods. CD42a positive PBMCs were determined as PBMC-platelet aggregates by flowcytometry. (*, $P < 0.05$; **, $P < 0.01$ compared to incubation with medium)

6.2.4 Atorvastatin inhibits primate platelet-PBMC aggregates after incubation with PAECs

The WT PAECs were pretreated with atorvastatin (5 μ M) for 30 minutes or 16 hours; without washing, primate platelets and PBMCs were added for another 8 hours co-incubation. Only after pretreated for 16 hours, atorvastatin inhibited platelet and PBMC aggregation after incubation with PAECs. This effect was not seen after pretreatment with atorvastatin for only 30 minutes or when mevalonic acid was added at the same time (Figure 6.2-4). This demonstrated that the inhibitory effect resulted from the presence of atorvastatin and needed longer incubation time to take effect.

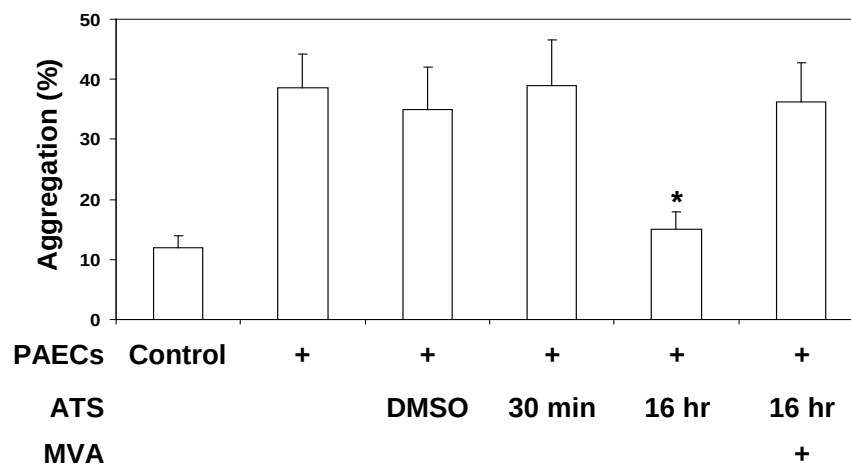


Figure 6.2-4 Atorvastatin inhibits primate platelet-PBMC aggregation after incubated with PAECs

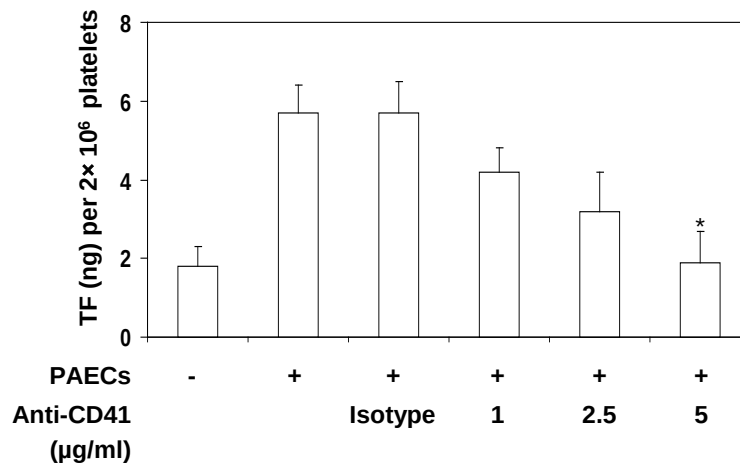
Primate platelets and PBMCs were pre-treated with atorvastatin (ATS) for 16 hours or 30 minutes in the presence or absence of mevalonic acid (MVA) for 30min. Thereafter, platelets and PBMCs were incubated with PAECs for 8 hours and harvested for analysis

of platelet-PBMC aggregation by flowcytometry. (*, $p < 0.05$ compared to incubation with PAECs alone).

6.2.5 Anti-CD41 antibody inhibited TF expression on platelets

We further investigated the role of membrane GPIIb/IIIa (CD41) in this system, which contributes to the stabilization of thrombus after platelets are activated. The experiments were repeated with the addition of anti-human CD41 neutralizing Abs. Anti-CD41 Abs inhibited TF expression on platelets after incubation with PAECs (Figure 6.2-5).

A



B

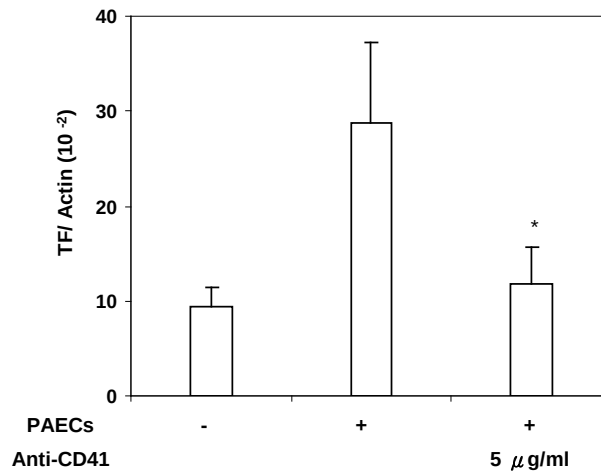


Figure 6.2-5 TF expression on platelets after incubated with PAECs is inhibited by anti-CD41 antibody.

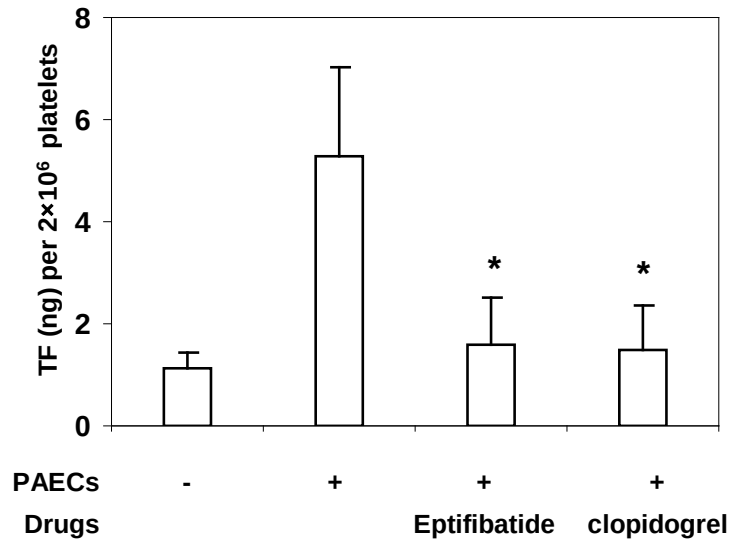
Primate platelets and PAECs were pre-treated with a series of concentrations of neutralizing Abs for 30 minutes at 4°C. Thereafter, platelets were incubated with PAECs and harvested for analysis by the recalcified clotting assay (A) and quantitative RT-PCR (B). (*: $p < 0.05$ compared to incubation with PAECs)

6.2.6 Eptifibatide and clopidogrel inhibit TF expression on platelets activated by PAECs

We went on to examine the effect of the anti-platelet agents, eptifibatide (2μg/ml) and clopidogrel (0.1μg/ml) on TF expression of platelets. The platelets were pretreated with a series of concentrations of eptifibatide and clopidogrel for 30min. Then they were co-incubated with PAECs without washing. The platelets were harvested for analysis of TF activity. Both eptifibatide and clopidogrel inhibited TF expression on platelets after

incubation with PAECs by recalcified clotting assay and quantitative RT-PCR (Figure 6.2-6).

A



B

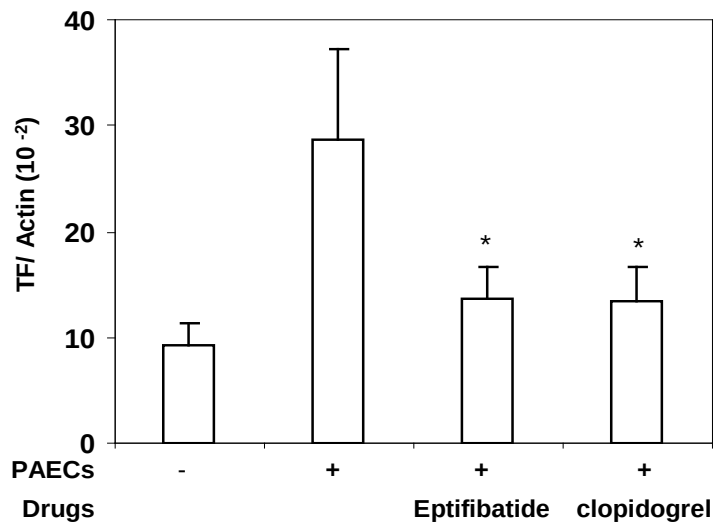


Figure 6.2-6 TF expression on platelets after incubation with PAECs is inhibited by anti-platelet agents.

Primate platelets and PAECs were pre-treated with eptifibatide and clopidogrel for 30 minutes at 4°C. Thereafter, platelets were incubated with PAECs for analysis by the

recalcified clotting assay (A) and quantitative RT-PCR (B). (*: $p < 0.05$ compared to incubation with PAECs)

6.3 DISCUSSION

Sensitized baboon serum with high titres of anti-nonGal Abs activated PAECs to express TF activity on PAECs independent of complement activation. This may contribute to the coagulopathy after transplantation of organs from GT-KO pigs with hCRP genes into baboons. By preventing TF expression, statins have anticoagulant effects. These results demonstrated that atorvastatin does not only inhibit functional TF activity but also the expression of TF mRNA when PAECs were activated with anti-nonGal Abs.

Although statin inhibits TF expression in some types of cells, the reports of its inhibitory effect on TF expression on platelets are scarce. We also demonstrated atorvastatin inhibited TF expression on platelets after incubation with PAECs. Since statin remained in the incubation system, the benefit could result from two possibilities. First, statin indirectly prevents TF expression on platelets by interfering with interactions between PAECs and primate platelets. This indirect action is less likely because it needs a longer incubation time for atorvastatin to take effect on PAECs. The time course showed that atorvastatin needed 16 hours incubation to inhibit TF up regulation on PAECs after incubation with baboon serum and to prevent lymphocyte proliferation after incubation with PAECs (Ezzelarab et al., 2008). In the present study, pretreatment with atorvastatin for 30 minutes was long enough to inhibit TF expression on platelets. The effect seems to result from the direct inhibition on platelets themselves. Recently, a novel NO-releasing statin inhibited the aggregation of platelets induced by collagen (Rossiello et al., 2005). Moreover, it also inhibited collagen-induced platelet P-selectin expression in whole blood as well as platelet adhesion to collagen-coated cover slips under flow conditions. This

finding is compatible with the result that TF activity of platelets after incubation with PAECs is inhibited when neutralizing anti-human P-selectin Ab is present (Figure 5.2-6).

My previous study showed that PAECs activated platelets and PBMCs to express TF activity. Similarly, PAECs also promoted the aggregation of platelets and leucocytes independent of XNA and complement. The aggregates play a central role in inducing a further burst of cellular interaction. This suggests that further manipulation of the immune response will not solve the coagulopathy completely. This study also showed atorvastatin was able to inhibit this aggregative reaction induced after incubation with PAECs.

Extensive evidence has shown that the anti-platelet agents, such as eptifibatide and clopidogrel, inhibit platelet aggregation in arterial ischemia or xenotransplantation models. Therefore, they confer anti-thrombotic activity (Herault et al., 1999; Savi et al., 1994)). However, a recent study indicated that the effect of clopidogrel is beyond inhibiting platelet aggregation and reduces circulating TF-procoagulant levels in patients with peripheral arterial disease (Rao et al., 2006). At the same time, plasma P-selectin level also declined. The mechanism of clopidogrel inhibiting circulating TF is attributed to downregulation of expression of P-selectin on platelets, inhibition of platelet-leucocyte aggregation and subsequently reduction of TF expression on leucocytes (Evangelista et al., 2005; Leon et al., 2004). Similarly, GPIIb/IIIa antagonists (tirofiban and eptifibatide) inhibit platelet aggregation and improve heart performance in an *ex-vivo* perfusion model by preventing intravascular thrombosis (Brandl et al., 2007) although direct evidence of

inhibition of TF-induced coagulation activation in humans with low-grade endotoxemia was not detected (Derhaschnig et al., 2003).

In the present study, we clearly showed eptifibatide and clopidogrel prevented TF activity on platelets after incubation with PAECs, which was unrelated to the involvement of leucocytes. These effects result from the inhibition of the autocrine feedback pathway of ADP and GPIIb/IIIa on platelets. These observations suggest eptifibatide and clopidogrel exert anti-thrombotic activity through a distinctive mechanism.

Anti-platelet agents have been used to try to resolve coagulopathy during xenotransplantation. In a hCD46-transgenic pig-to-baboon cardiac transplantation model, warfarin and low-molecular weight heparin had no impact on graft survival, with no reduction in fibrin deposition and platelet thrombi. The combination of aspirin and clopidogrel neither prolonged graft survival nor inhibited thrombosis in cardiac xenografts (Schirmer et al., 2004). However, clopidogrel exerted some systemic inhibition on platelet aggregation. A high dosage of a GPIIb/IIIa antagonist prolonged graft survival and decreased platelet aggregation in a rodent model (Candinas et al., 1996). Eptifibatide also effectively prevented platelet aggregation in a primate study (Alwayn et al., 2000). Although the impact on graft survival has hitherto been disappointing, these observations suggest that anti-platelet agents are sufficient to prevent platelet aggregation in the circulation. Moreover, the effect of anti-platelet agents depends on the intensity of stimulation (Ciborowski et al., 2008), which implies that would inhibit the generation of thrombin and activation of ECs will allow these drugs to be more beneficial.

Although the mechanisms of statins in xenotransplantation are not well-defined, its pleiotropic effects offer the opportunity to tackle the complex problems seen in xenograft rejection. It will be noteworthy to examine the effect of atorvastatin in animal studies. The failure of anti-platelet drugs in previous studies may be because the immune response was not well-controlled at that time and the intensity was so strong to overshadow the benefit of anti-platelet agents. With progress in the genetic modification of pig donors, the immune response has been significantly alleviated by deleting antigen and expressing a hCRP. This pharmaceutical systemic approach may offer additional survival benefits by preventing platelet activation.

CHAPTER 7

THE ROLE OF RECIPIENT TF IN PIG-TO-

BABOON ORGAN

XENOTRANPLANTATION

7.1 INTRODUCTION

Intravascular thrombosis or systemic coagulation disorders during AHXR has been a major hurdle to successful xenotransplantation. In the model of heterotopic cardiac xenotransplantation from GT-KO swine to baboons, all grafts failed due to the intragraft exhibition of TM with platelet-rich fibrin thrombi in the microvasculature, myocardial ischemia and necrosis, and focal interstitial hemorrhage. The development of TF was in parallel with the activation of ECs in the grafts, presented with the upregulation of TF and vWF and down-regulation of CD39. This implied TM is initiated by the expression of TF. Previous studies stressed that the origin of TF derived from the activation of ECs in response to XNA and complement activation. However, the coagulation disorder developed not only in the graft, several studies reported AHXR is associated with systemic coagulation cascade activation, consistent with non-physiologic DIC.

This systemic consumptive disorder is compatible with DIC seen in varying underlying conditions, including endotoxemia, cardiac surgery and cancer (Langer F, 2008; Mackman N, 2007). The pathophysiology of DIC may be associated with liberation of constitutive TF into the blood and inducible TF expression on leucocytes and other cells.

On the other hand, a similar situation occurs in islet transplantation by the method of portal vein infusion. Many transplanted islets are lost immediately after transplantation due to the instant blood-mediated inflammatory reaction (IBMIR). IBMIR is associated with the presence of TF expression on endocrine cells and subsequent contact with portal blood (Bennet et al., 2000, Johansson et al., 2005). This observation was demonstrated in

an animal study. Berman et.al showed that interference with TF by using a humanized anti-TF Ab leads to prolonged and/or enhanced islet allograft function in an a non-human primate model (Berman et al., 2007).

Furthermore, as demonstrated in Chapter 3, my *in vitro* study showed that PAECs can activate platelets and PBMCs to express TF activity. Therefore, we hypothesized that TF activity was not only expressed on ECs of the grafts. Recipient platelets and leucocytes also expressed TF activity in the systemic circulation. This presentation may be associated with the development of systemic consumptive coagulopathy during AHXR.

7.2 RESULTS

7.2.1 Survival of animals

The survivals of all animal were described in detail (Table 7.2-1). The liver allograft recipient survived for 28 days uneventfully and was euthanized by protocol. In the HAR group, the baboon receiving a kidney graft developed acute renal failure (rapidly increased serum creatinine level) within 24 hours; at euthanasia, the graft revealed gross thrombotic changes compatible with features of typical HAR. The liver xenograft was euthanized 5 hours after implantation, although the graft was not grossly thrombotic.

In non-HAR kidney group, HAR was averted by either using the grafts from genetically-modified pigs or by administering immunosuppressants to inhibit the humoral response. All 6 recipients of kidney grafts with immunosuppressants died of consumptive coagulopathy and survived 6 to 15 days (median: 10 days). The other two recipients without immunosuppressant died of transplant-unrelated causes 2 and 4 days after transplantation (iatrogenic fluid overload was suspected in B4407). At that time, hematological and biochemical profiles were within normal limits and there was no significant abnormal finding at autopsy. Platelet counts and serum creatinine levels in baboon recipients of pig kidney grafts were presented in Figure 7.2-1 .

After liver xenotransplantation, coagulation disorder presented by severe thrombocytopenia and thrombin formation developed in all recipients of liver grafts within 5 hours. The platelet counts dropped from 270 ± 60 to $50 \pm 20 \times 10^3/\mu\text{L}$ and D-dimer

increased from 1.25 ± 0.55 to 2.12 ± 0.06 $\mu\text{g/ml}$ with fibrin formation in the graft (Figure 7.2-2). The four recipients of liver grafts died of consumptive coagulopathy and survived 4 to 7 days (median: 6 days). At euthanasia, the liver grafts grossly presented scattered thrombotic patches (necrotic part) in the liver surface.

	Group	Baboon No	Organ	Type of pig	Immunosuppressant	Survival (Days)	Cause of death
Allograft	I (n=1)	B3408	Liver	Baboon liver	FK based	28	Protocol euthanized
HAR	II (n=2)	B3807	Kidney	WT	None	<1	Graft necrosis
		B16907	Liver	WT	None	<1	Protocol euthanized
Non HAR	III (n=8)	B4407	Kidney	GT-KO, MCP	None	2	CC
		B4507	Kidney	GT-KO, MCP	None	4	Unknown
		B3907	Kidney	WT	MMF based	6	Unknown
		B18308	Kidney	GT-KO	MMF based	7	CC
		B4207	Kidney	GT-KO, MCP	MMF based	10	CC
		B4307	Kidney	GT-KO, MCP	MMF based	10	CC
		B16307	Kidney	GT-KO, MCP	MMF based	10	CC
		B16007	Kidney	GT-KO, MCP	MMF based	15	CC
	IV (n=4)	B3108	Liver	GT-KO	FK based	6	CC
		B3208	Liver	GT-KO, MCP	FK based	4	CC
		B7708	Liver	GT-KO, MCP	FK based	7	CC
		B7808	Liver	GT-KO, MCP	FK based	6	CC

Table 7.2-1 The graft types, immunosuppressants, survival time and causes of death in each group of animal studies.

CC: consumptive coagulopathy

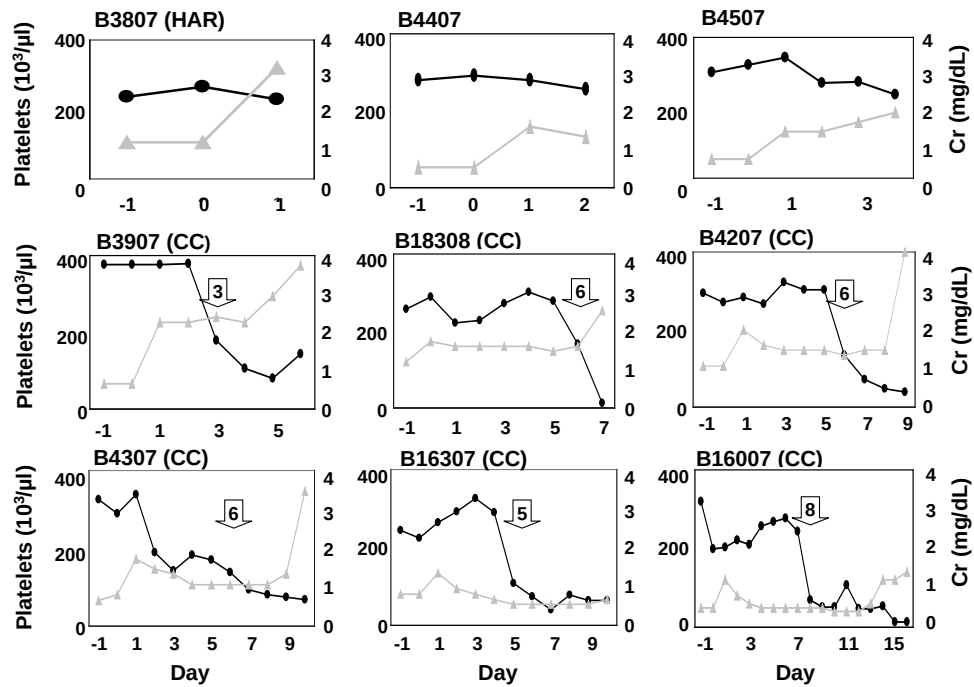


Figure 7.2-1 Platelet counts and serum creatinine levels in baboon recipients of pig kidney grafts.

B3807 developed HAR with a rapidly increasing creatinine level, but its platelet count remained unchanged. Two baboons (B4407, B4507) died of causes unrelated to AHXR or CC. The remaining 6 baboons developed features of CC (defined in the text). Arrows with numbers indicate the day on which CC was initially documented. (The day of kidney Tx was designated as day 0). HAR = hyperacute rejection; CC = consumptive coagulopathy, black line: platelet counts, gray line: serum creatinine level.

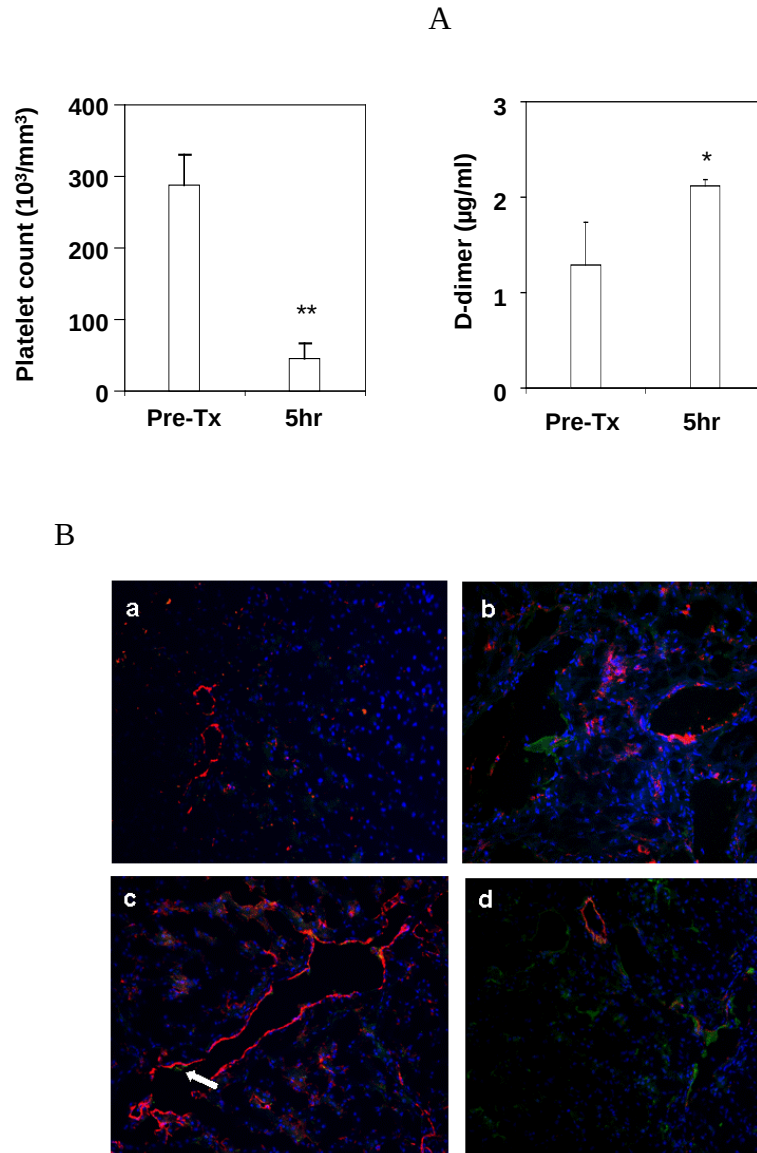


Figure 7.2-2 Consumptive coagulopathy developed immediately after liver xenotransplantation.

- A. Platelet counts decreased (left panel) and the level of D-dimer increased immediately after reperfusion (n=4).
- B. Immunofluorescence staining shows fibrin (green) deposits in the vessels of the graft.
- a. pre-perfusion, b. HAR in B16907, c. 2 hours after reperfusion in B7708 d. 7 days after transplantation in B7708. (Red: CD31 for ECs, arrow: fibrin deposition)

7.2.2 Platelets and PBMCs express baboon TF activity after xenograft transplantation

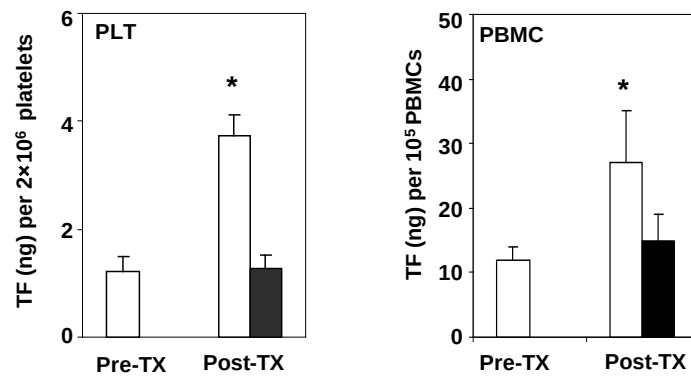
Platelets and PBMC were isolated from the baboon's peripheral blood before and after transplantation. TF activity on platelets and PBMC was determined by the recalcified clotting assays, quantitative RT-PCR and flowcytometry. Before operation, no TF activity was detected on platelets and PBMCs. In the liver allograft group (n=1), TF activity was not detected on platelets and PBMCs throughout follow-up for 28 days after transplantation.

7.2.2.1 HAR group

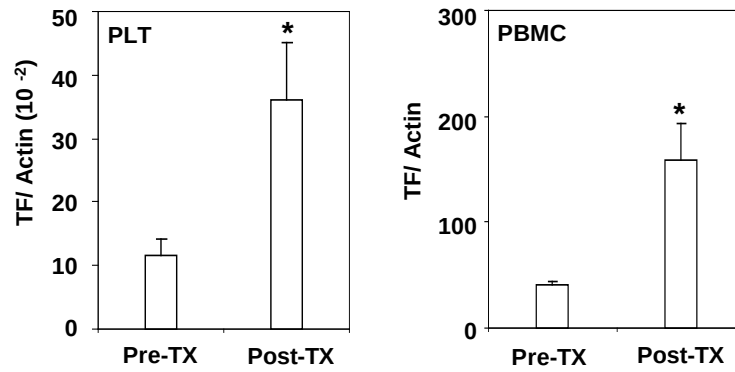
TF activity was expressed on platelets and PBMCs after liver or kidney pig xenograft transplantation by the recalcified clotting assay. TF activity on platelets and PBMCs could be inhibited by anti-human TF Ab, so these TF activities were derived from baboon, but not porcine origin (Figure 7.2-3A). The mRNA levels of TF were also higher in platelets and PBMCs after transplantation compared to pre-transplantation by quantitative RT-PCR (Figure 7.2-3B). Furthermore, the surface expression of TF was confirmed by flowcytometry (Figure 7.2-3C).

Since TF activity on PBMCs was much larger than on platelets, evidenced by RT-PCR and clotting assay, TF expression was expressed on the surface of PBMCs, not from platelets, which aggregated with PBMCs.

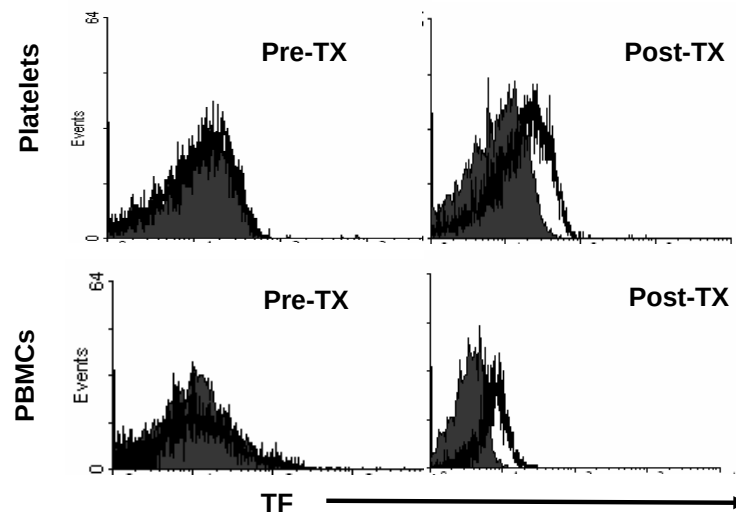
A



B



C



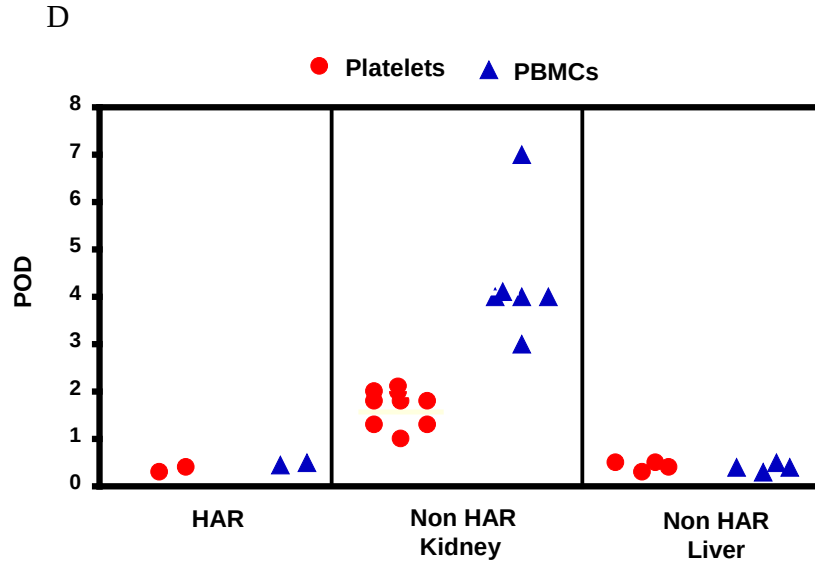


Figure 7.2-3 Platelets and PBMCs expressed TF after xenotransplantation.

A,B,C. The expression and activity of platelets and PBMCs were determined by the recalcified clotting assay in all recipients (A), quantitative RT-PCR in HAR baboon (B3807) (B) and flowcytometry in all baboons (representative by B4207) (C) before and after xenotransplantation. (Black bar: the cells were pre-incubated with anti-human TF Ab for 30 minutes at 4°C.)

D. The time when PBMCs or platelets to express TF activity was measured by the recalcified clotting assay. (POD: post-operative day)

7.2.2.2 Non-HAR kidney group

TF activity on platelets was detected on postoperative day (POD) 1 or 2 (n=8) by recalcified clotting assay and flowcytometry. In contrast, TF activity on PBMCs was not documented until POD 4 or 7 (median =5, n=6) exclusive of two baboons that died of unknown causes.

7.2.2.3 Non-HAR liver group

Similar to the HAR group, TF activity and expression on platelets and PBMCs were detected 5 hours after xenotransplantation. The days on which TF was expressed on platelets or PBMCs were summarized in Figure 7.2-3D.

7.2.3 The expression of TF on PBMCs is associated with the onset of consumptive coagulopathy

We further examined 7 baboons that developed consumptive coagulopathy to address the relationship between the expression of TF on PBMCs and the onset of consumptive coagulopathy. TF activity on PBMCs was usually expressed 2 days before the onset of consumptive coagulopathy in 5 baboons of kidney graft group. The onset of consumptive coagulopathy was highly correlated with TF expression on PBMCs (Figure 7.2-4).

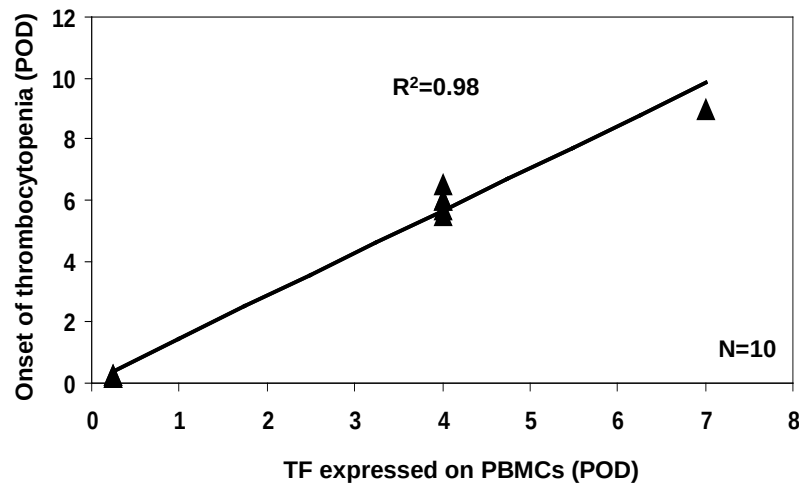


Figure 7.2-4 TF expression on PBMCs predicted the onset of consumptive coagulopathy.

The relationship between TF activity on PBMCs. TF activity was detected by recalcified clotting assay. The onset of thrombocytopenia was defined as 30% drop in platelets counts (POD: post-operative day).

7.2.4 Grafts remain functioning during consumptive coagulopathy

When the recipient developed consumptive coagulopathy, the function of the graft usually remained functioning. On the date of onset of consumptive coagulopathy, the serum creatinine levels of baboons who died of consumptive coagulopathy was $0.8 \pm 0.4 \text{ mg\%}$ (n=6) with adequate urine output. The serum creatinine level then increased, presumably due to bleeding and hypovolemia until they required euthanasia.

Similarly, the peak aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were on POD1 after liver xenograft transplantation and gradually returned to a level of about 1000(U/L). At the same time, the level of total bilirubin still remained

about 2.3 ± 1.4 mg% (n=4) until the day on which the baboons were critical. The parameters of the hematological and liver function tests are presented in Figure 7.2-5. Furthermore, the gross color of the kidney xenografts was pink unlike typical HAR, which were thrombotic and necrotic. Most of liver xenografts also showed non –necrotic appearance with patchy ischemic change. The coagulation function and complement profiles were presented elsewhere in detail (Ekser B, Transplantation submitted).

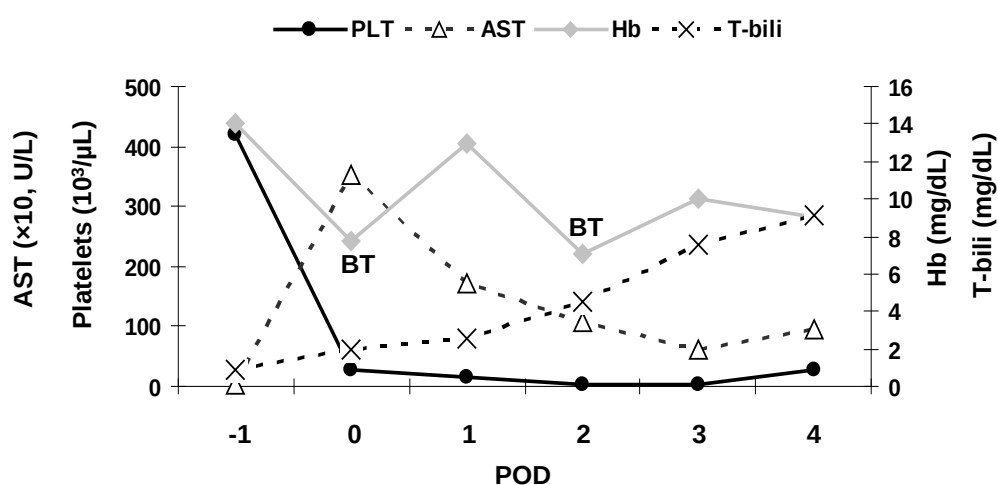


Figure 7.2-5 Hematological and liver function tests in baboons who underwent liver transplantation.

The data were presented as mean of values in 4 baboons. PLT: Platelet, Hb: hemoglobin, T-bili: total bilirubin. BT: blood transfusion.

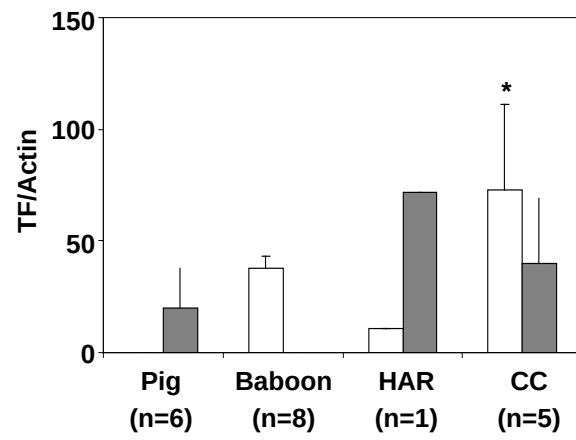
7.2.5 Recipient TF expression in the grafts

Baboon TF activity was assessed by quantitative RT-PCR. The kidney tissues were collected at the time of euthanasia; the liver tissues were collected at the time of reperfusion and euthanasia. Baboon TF mRNA was detected in the porcine grafts, which was even higher than in native baboon kidneys when normalized by β actin (Figure

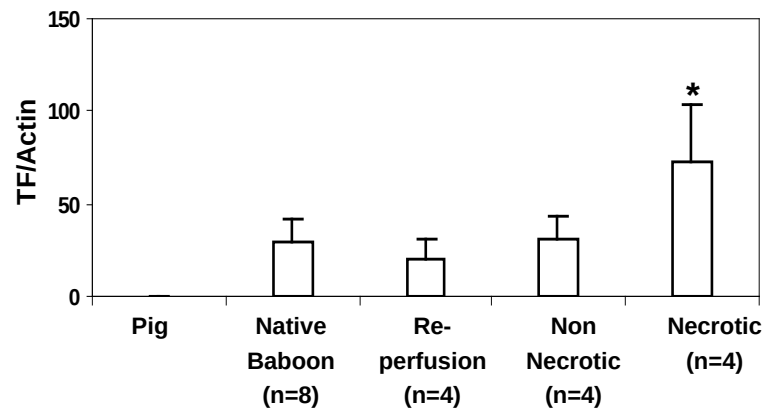
7.2-6A). Baboon TF mRNA was detected in porcine liver xenografts and was significantly higher in tissues with thrombotic change compared to immediately after reperfusion but was not higher in the tissues without thrombotic change (Figure 7.2-6B).

Immunofluorescence staining revealed that large numbers of platelets deposited in the thrombi and expressed TF when AHXR developed. At the same time, PBMCs also expressed TF on the surface (Figure 7.2-6C).

A



B



C

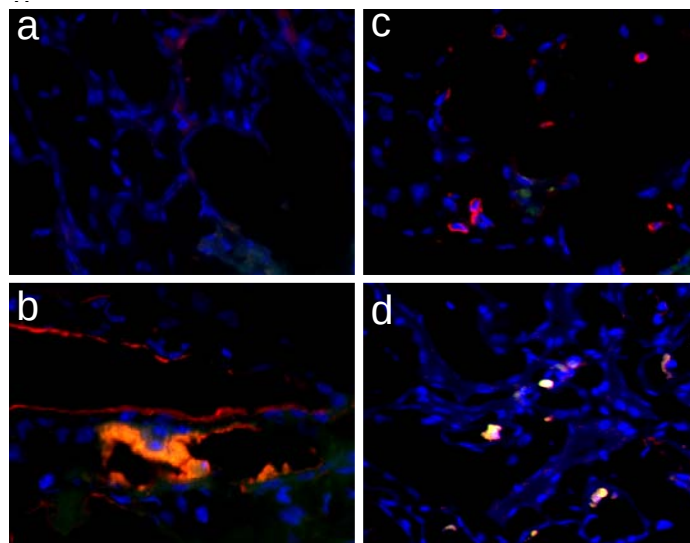


Figure 7.2-6 Baboon TF is expressed in porcine xenografts during consumptive coagulopathy.

- A. TF expression in the graft was determined by quantitative RT-PCR with baboon (white) and pig (gray) TF primers. During consumptive coagulopathy (CC), baboon but not porcine TF was significantly expressed in the kidney grafts (* $p < 0.05$ compared to naïve baboon, CC: baboons who developed CC).
- B. TF expression in the graft was determined by quantitative RT-PCR with porcine TF primer after liver xenotransplantation. At euthanasia, liver xenografts grossly appeared heterogenous demaceration including thrombotic change (necrotic) and normal tissue (non-necrotic) (* $p < 0.05$ compared to naïve baboon, CC: baboons who developed CC).
- C. Platelets (a,b) (CD41,red) plugged in the thrombi of graft and PBMCs (c,d) (CD45, red) expressed TF (green) after AHXR (a,c: pre-transplant, b,d: post-transplant in B18307).

7.2.6 Recipient TF expression in the extra-graft organs

Since circulating inflammatory cells (platelets and PBMCs) expressed TF after xenotransplantation, they would induce systemic TF expression not only in the grafts but also in the other organs. The native livers from the recipients for liver xenotransplantation and hearts from the donors for baboon liver allotransplantation were used as pre-operative control. After kidney xenotransplantation, the level of TF mRNA in the livers and hearts were significantly increased by quantitative RT-PCR (Figure 7.2-7).

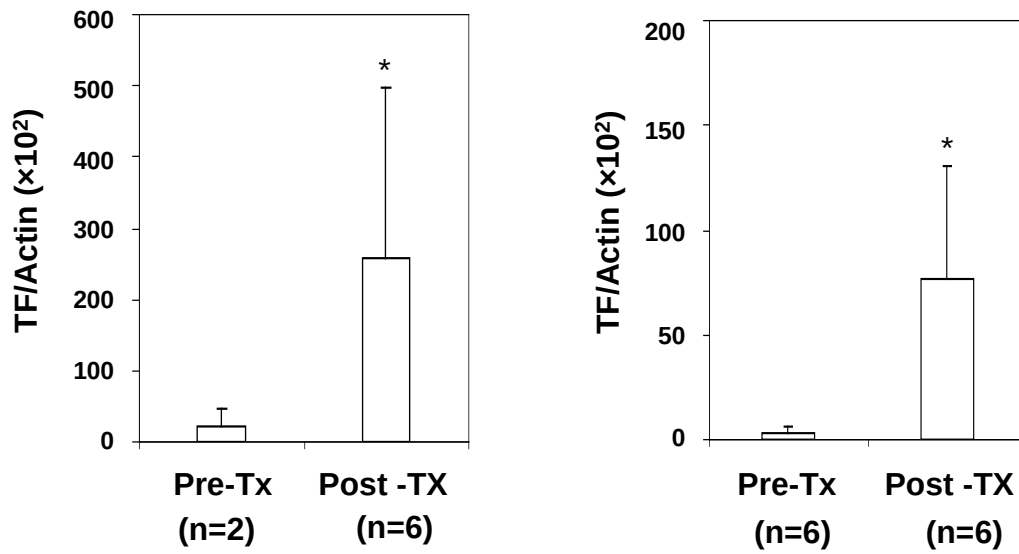


Figure 7.2-7 TF expression is increased in extra-graft organs after kidney xenotransplantation.

TF expression in the hearts (left) and liver (right) was measured by quantitative RT-PCR.

7.2.7 Histopathology in AHXR-kidney group

The histopathological findings at the time of graft excision or recipient euthanasia of 5 kidney graft recipients that developed HAR and consumptive coagulopathy are summarized in Table 7.2-2. Of importance, 3 of 5 showed the features of minimal humoral response (Figure 7.2-8). There was only minimal IgM deposition without the deposition of IgG, C3, or infiltration of macrophages, B and T cells. Deposition of fibrin and platelets was indeed found.

	IgM	IgG	Fibrin	C3	MΦ	PMN	B cells	T cells
B3807	(++)	(++)	(+++)	(+++)	(++)	(++)	(+)	(+)
B3907	(+)	(+)	(+++)	(--)	(--)	(--)	(--)	(--)
B18308	(+)	(+)	(+++)	(--)	(--)	(--)	(--)	(--)
B4207	(+/-)	(--)	(++)	(--)	(--)	(--)	(--)	(--)
B4307	(+/-)	(--)	(++)	(--)	(--)	(--)	(--)	(--)
B16307	(+/-)	(--)	(+++)	(--)	(--)	(--)	(--)	(--)
B16007	(++)	(++)	(+++)	(++)	(--)	(--)	(--)	(--)

Table 7.2-2 Histopathology of kidney grafts that developed hyperacute rejection and consumptive coagulopathy

(--) none; (+/-): minimal; (+) mild; (++) moderate; (+++) severe.

MΦ = macrophages; PMN = polymorphonuclear neutrophils

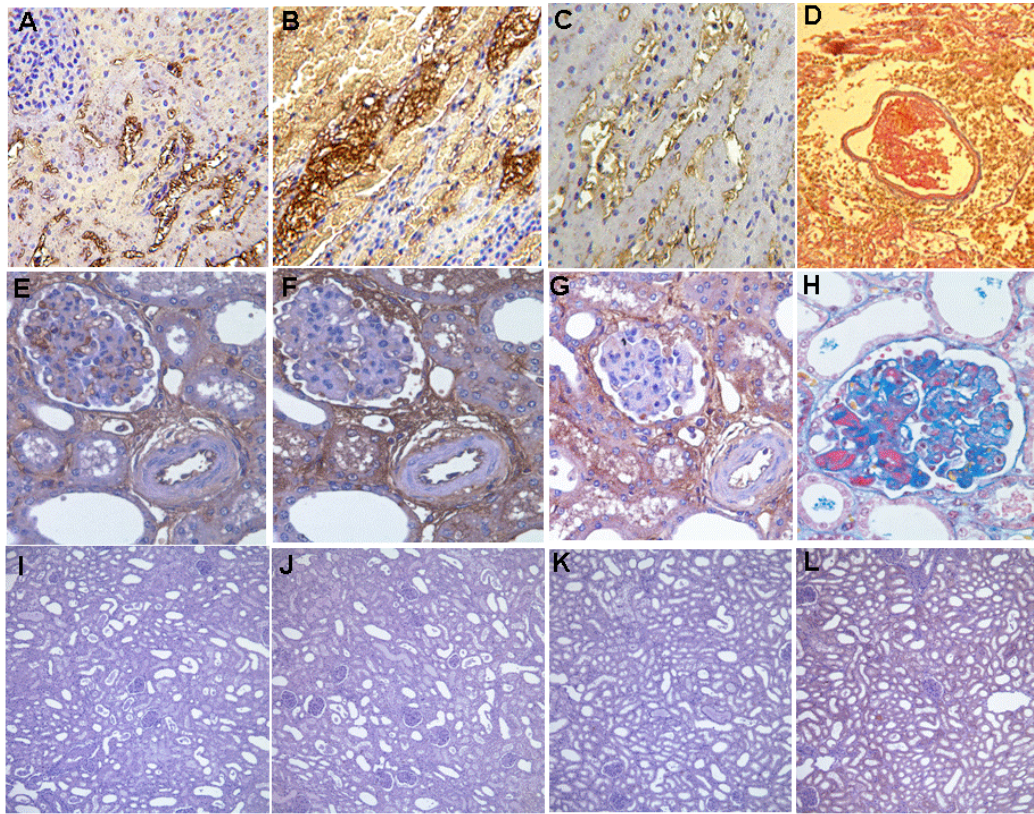
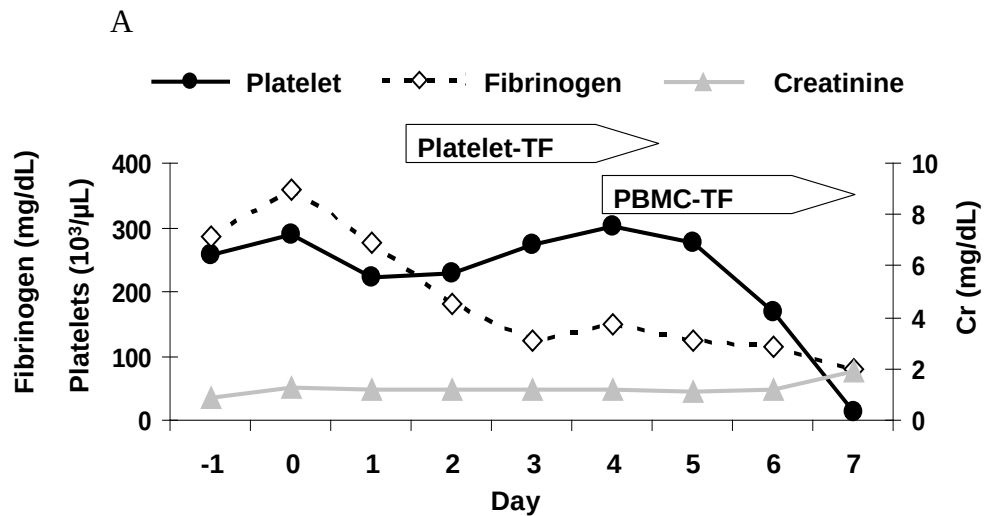


Figure 7.2-8 Histological features of kidney grafts showing minimal immune response.

Deposition of IgM (brown in A), IgG (brown in B), C3 (brown in C) and fibrin (red in D) is presented in HAR kidney graft (B3807). In contrast, the grafts in AHXR group (B4207) are minimal deposition of IgM (E), negative for IgG (F), C3 (G), macrophage (CD68) (I), B cell (CD20) (J), CD4 (K) and CD8 (L) T lymphocytes with some fibrin deposition (H).

7.2.8 Clinical course in AHXR-kidney group

In summary, the clinical courses of kidney graft recipients that developed consumptive coagulopathy, were associated with the expression of TF on recipient platelets and PBMCs (Figure 7.2-9A). TF expression on platelets occurred early after the kidney grafts were implanted. Thereafter, serum fibrinogens started to drop after TF expression on platelets but before on PBMCs. Then PBMCs were activated to express TF activity later after transplantation, which was associated with the development of consumptive coagulopathy that presented with severe thrombocytopenia, anemia, and exhaustion of coagulation factors. Meanwhile, the renal grafts were still functioning with adequate urination. In B16007 baboon, the serum creatinine remained in normal range for another 7 days after thrombocytopenia (Figure 7.2-9B). Finally, recipients succumbed to systemic complications.



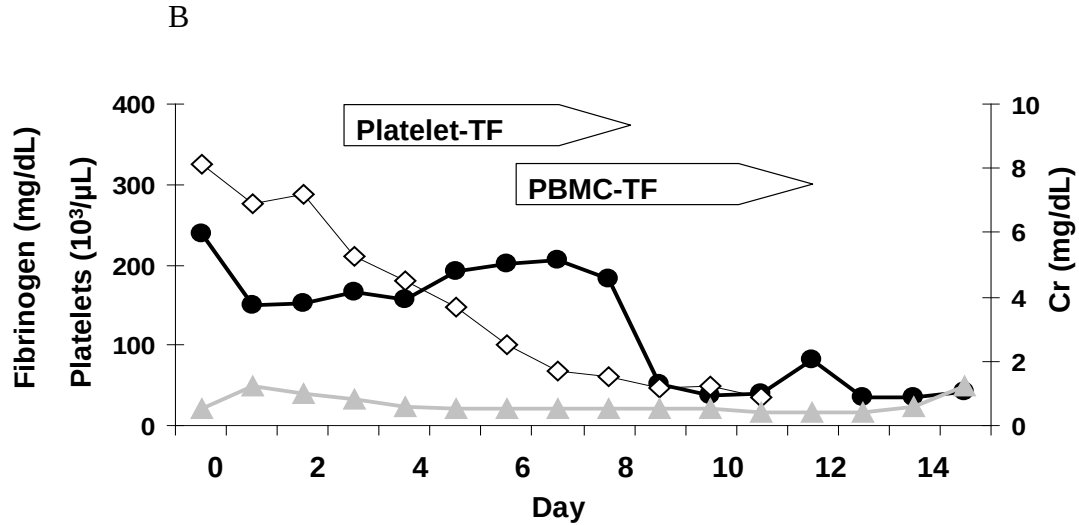


Figure 7.2-9 Clinical manifestations of kidney graft recipients who developed consumptive coagulopathy.

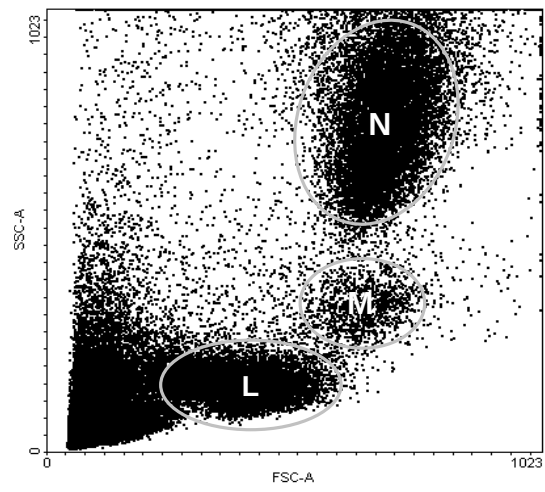
- A. The data are presented as mean, representative of 4 kidney graft recipients (B4207, B4307, B16307, and B18308). (Cr:creatinine, POD: post-operative day)
- B. The data is representative of B16007 with the longest survival.

7.2.9 Platelet and leucocyte aggregates during consumptive coagulopathy

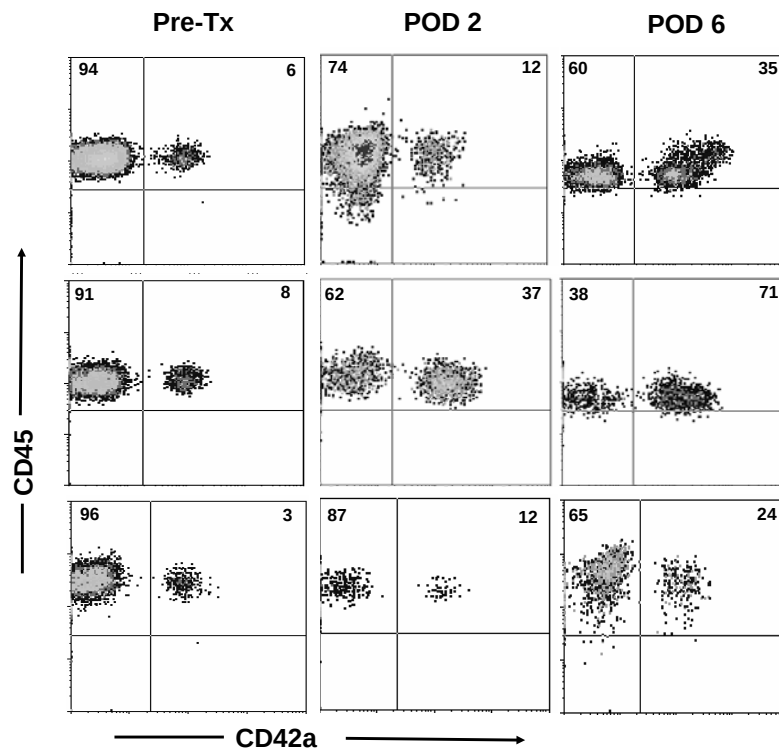
We went on to investigate the interaction between platelets and leucocytes during the development of consumptive coagulopathy. Leucocytes and platelets were isolated from 0.5 ml of peripheral blood with 5 ml ACK (ammonia chloride-potassium) buffer (Appendix 8) for lysis of red blood cells. The phenotype of leucocytes was confirmed by the marker of CD45 and dot plot in flowcytometry. Aggregation between platelets and leucocyte was determined by the percentage of CD42a expression in the leucocyte gate. The platelet and granulocyte, monocyte and lymphocyte aggregates were significantly

greater on post-operative day 2, on which TF was expressed on platelets after kidney xenotransplantation, compared to before transplantation. (Figure 7.2-10A,B). In addition, the platelet-leucocyte aggregation dramatically increased when leucocytes expressed TF activity (Figure 7.2-10B,C).

A



B



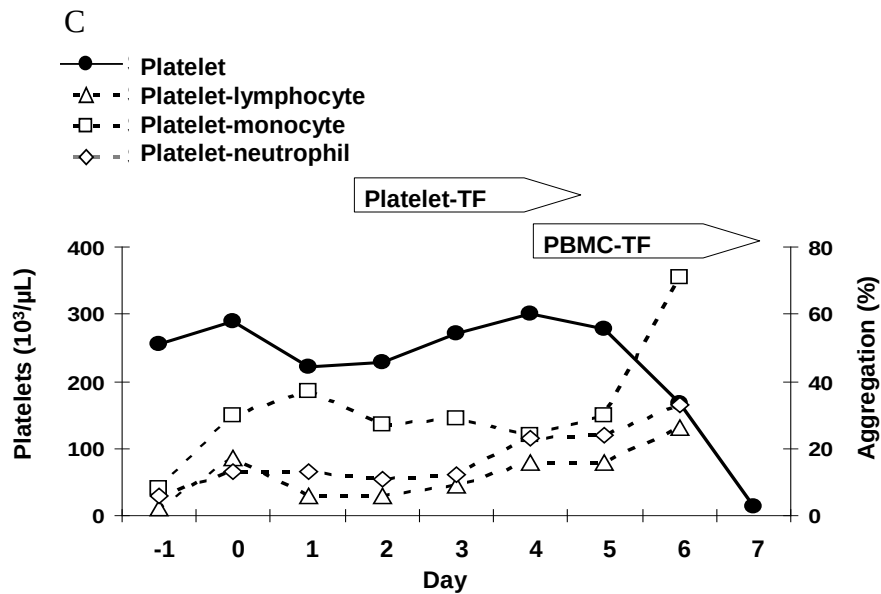


Figure 7.2-10 Platelet and leucocyte aggregation increases after kidney xenotransplantation.

Leucocytes were isolated from baboon's peripheral blood before and after kidney xenotransplantation. Thereafter, leucocytes were stained by CD42a as platelets marker. The phenotype of leucocytes was confirmed by the expression of CD45. Granulocytes, monocytes and lymphocytes were determined by dot plot of flowcytometry. CD45 and CD42a double positive cells were designated as platelet-leucocyte aggregates.

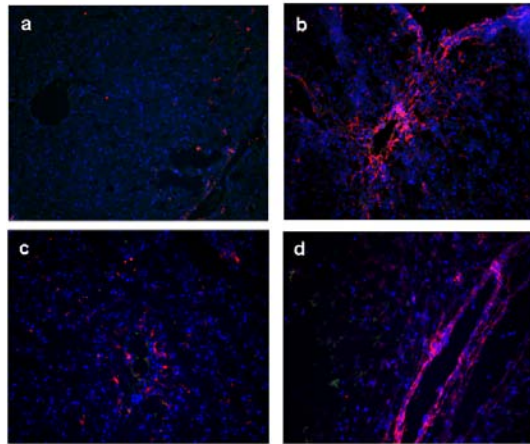
- A. In dot plot, there are 3 distinctive groups of cells including lymphocytes (L), monocytes (M) and granulocytes (G).
- B. The percentages of CD42a-positive cells in the granulocyte gate (upper), the monocyte gate (middle) and the lymphocyte gate (lower) in pre-operative, POD2 (TF expression on platelets) and POD6 (the onset of thrombocytopenia). The percentage of cells in the quadrant is indicated. (POD: post-operative day)

C. The clinical courses of platelet-leucocyte aggregation, TF expression and consumptive coagulopathy in B18308.

7.2.10 Consumptive coagulopathy develops before the activation of PAECs after liver xenotransplantation

To further investigate what cells (donor PAECs or recipient inflammatory cells) are the first to initiate the coagulation disorder in xenotransplantation, we examined the activation of PAECs by the expression of VCAM-1 (CD106) and TF. By 2 hours after reperfusion when consumptive coagulopathy was developing, the expression of VCAM-1 and TF on PAECs of grafts was still minimal by immunofluorescence staining (Figure 7.2-11).

A



B

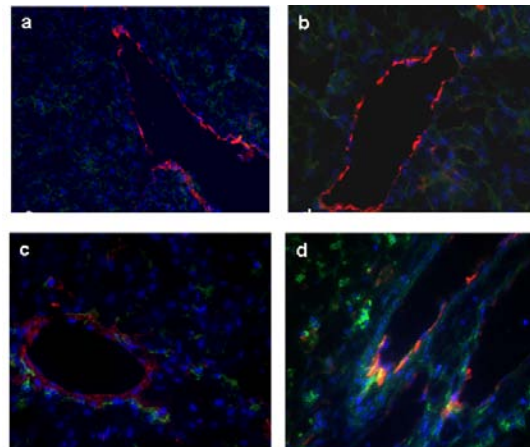


Figure 7.2-11 The expression of VCAM-1 and TF in liver xenografts is minimal 2 hours after reperfusion.

A. The expression of VCAM-1 (red).

B. The expression of TF (green) and CD31(red).

a: pre-perfusion, b: HAR in B16907, c: 2 hours after reperfusion in B7708; d: 7 days after transplantation in B7708.

7.3 DISCUSSION

Although the survival of xenografts has been extended after the transplantation of hearts from GT-KO pigs into baboons, the results of kidney and liver transplantation are discouraging. In the present study, the median survivals of kidney and liver grafts from GT-KO/MCP pigs were 10 and 6 days respectively. Interestingly, they shared the common complication and causes of mortality despite the different time of onset. Most of them died of consumptive coagulopathy associated with severe thrombocytopenia, anemia and bleeding tendency although the organs still remained functional, evidenced by adequate urine amount and biochemistry parameters. Importantly, the histology of the grafts showed only a minimal immune response without deposition of IgG and infiltration of macrophages, B and T cells (unlike that seen in typical AHXR) in kidney xenograft model. These observations suggest that the development of consumptive coagulopathy was dissociated from the presence of an immune response.

Extensive evidence demonstrated that coagulopathy in AHXR is initiated by the expression of TF. However, the sources of TF still remain elusive. One concept is that ECs of the graft, activated by the binding of XNA and/or activation by complement, express TF activity and trigger coagulation. The activated ECs and the generated thrombin subsequently activate platelets, leucocytes and other inflammatory cells to form a vicious circle. The other concept is that inflammatory cells, especially platelets and mononuclear cells, activated by soluble inflammatory mediators or other immune response-independent pathway in the grafts might stimulate coagulation via expression of TF (Blakely et al., 1994). In the present study, we first demonstrated that baboon

platelets and PBMCs were activated to express TF activity in pig-to-primate xenotransplantation. TF activity on platelets was expressed early after the xenografts were implanted and the timing was unaffected by graft types and immunosuppressive regimens.

Whether it is donor ECs or recipient platelets that are activated earlier to express TF after xenotransplantation still required investigation. We believe that TF expression on platelets precedes the activation of PAECs in the graft and results from humoral immune response-independent factors because of the following reasons. 1. Co-stimulatory blockade, anti-CD154 monoclonal Ab was routinely used in this series. The recipients were not sensitized immediately after transplantation. 2. Complement activity was regulated by the expression of MCP or eliminated by the administration of CVF (evidenced by the CH50 test). 3. Importantly, the histology of the grafts showed minimal evidence of an immune response, without deposition of IgG and infiltration of macrophages, B and T cells in kidney xenograft model. In addition, the kidney and liver xenografts were still functioning after the development of consumptive coagulopathy. For example, in the baboon (B16007), the serum creatinine levels remained in normal range for 7 days after the onset of severe thrombocytopenia.

More importantly, in liver xenograft model, platelets were activated to express TF before graft ECs expressed VCAM-1 and TF. These results indicate that activation of on platelets may not be associated with humoral rejection but initiated by other non-immunological factors, such as molecular incompatibilities. These findings are compatible with my previous *in vitro* finding that PAECs can activate primate platelets

and monocytes to express TF independent of a humoral immune response (Chapter 3). These observations were also found in other models by transplanting guinea pig cardiac xenografts into rats treated (Blakely et al., 1994). In HAR model, activation of donor ECs developed immediately after transplantation. In contrast, in a delayed humoral rejection model by the use of CVF, the expression of TF on the recipient infiltrating mononuclear cells was detected 12 hr after transplantation, which was prior to the expression of TF on donor ECs.

In addition to platelets, baboon PBMCs were activated to express TF activity. The onset of consumptive coagulopathy was associated with the development of TF expression on PBMCs in the non-HAR kidney and liver graft models. This coincidence is similar to the development of DIC in sepsis, in which inducible TF is predominantly expressed by monocytes and macrophages, as was shown in patients with severe bacterial infection (Schouten et al., 2008). Furthermore, a recent study indicated that leucocytes (dendritic cells) are the primary source and determine the extent of disseminated coagulation activation during exacerbation of inflammation (Niessen et al., 2008).

The critical question is what factors activate PBMCs to express TF activity and induce consumptive coagulopathy. There are three possible explanations. First, it has been previously thought that activated PAECs activated PBMCs to express TF via P- and E-selectin or thrombin, which is formed during PAEC activation and is a potent stimulator of PBMCs. This process is mainly involved with the immune response and subsequent activation of PAECs. Second, like platelets, PBMCs are activated to express TF through a

humoral response-independent pathway. Circulating PBMCs are directly activated when in contact with graft ECs in the graft. Third, TF expression of PBMCs is induced by activated platelets, which have been activated at earlier-time points after xenotransplantation. Activated platelets stimulate leucocytes to express TF activity via interaction between P-selectin and PSGL-1. Importantly, in the present study, we demonstrated that serum level of fibrinogen started to drop, concomitant with TF expression on recipient platelets. Moreover, platelets and PBMCs progressively aggregated until consumptive coagulopathy developed. In addition, the histopathological findings of grafts showed a minimal immune response with functioning. These observations suggest that TF expression on platelets plays an initial role to trigger the formation of thrombin. As a result, activated platelets and thrombin are potent stimulators to activate PBMCs or ECs, contributing to the development of consumptive coagulopathy during AHRX.

In addition, we found that thrombocytopenia developed earlier than the activation of ECs (by the expression of TF, VCAM-1) in the grafts of liver xenotransplantation. The rapidity of development of thrombocytopenia was not alleviated by the transplantation of a liver from a pig that did not express Gal but did express a hCRP, nor by immunosuppressive therapy. These results suggest it is recipient TF that initiates the consumptive coagulopathy that develops after organ xenotransplantation and results from a non-immune response.

Although this study elucidates that TF expression on platelets and PBMCs is associated with the development of consumptive coagulopathy, whether TF expression on pro-inflammatory cells in the circulation plays an exclusive role in this process requires further investigations. An *in vivo* study of using the infusion of anti-TF Ab or of pigs transgenic for TFPI to block the activity of TF would allow investigation of whether the survival of grafts will be extended or the development of consumptive coagulopathy will be attenuated.

In summary, we are the first to clarify that TF expression on recipient platelets and PBMCs plays a pivotal role in the induction of coagulopathy in pig-to-baboon xenotransplantation. This TF expression and activity seem to be unrelated to the immune response since they occur even when the response of XNA and activity of complement are minimal. This suggests that other non-immunological factors should be investigated. Hopefully, this can be manipulated either by genetic modification or systemic medication therapy. Moreover, further manipulation of the immune response, with the risk of infection and other complications, will not completely solve the challenge of xenograft coagulopathy.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE

DIRECTIONS

Extensive evidence has supported the view that thrombosis leads to the loss of both whole organ and islet xenografts in the pig-to-primate model. The mechanisms of the coagulation disorders seen in xenotransplantation are involved with complex immunologic and non-immunological reactions. Many novel approaches have not been entirely successful to lead to a significant advance. An understanding of the mechanisms is mandatory for further manipulation, which would allow xenotransplantation to advance to clinical trials.

8.1 IMMUNOLOGIC MECHANISMS CONTRIBUTING TO COAGULOPATHY DURING XENOTRANSPLANTATION

The interactions between porcine ECs, platelets, and other blood cells are at the nexus of a complex network that contributes to coagulation during AHXR (Figure 8.1-1). According to the standard paradigm, the process is initiated by the immune response against the graft, such that activation of PAECs caused by XNA with complement leads to expression of donor TF that triggers the coagulation cascade. In the present study, we demonstrated that natural or elicited anti-nonGal Abs induce PAECs to express TF activity via a complement-independent pathway. Together with loss of anticoagulant regulators, such as TFPI, thrombomodulin, or heparan sulfate, or through fgl-2 conversion, thrombin and fibrin are deposited in the graft. This detrimental process intrinsically involves potent platelet activation, which results from activated ECs or consequent formation of thrombin/fibrin. At the same time, the loss of ecto -NTPDases and failure of enzymatic degradation of ADP aggravates the activation and aggregation of platelets.

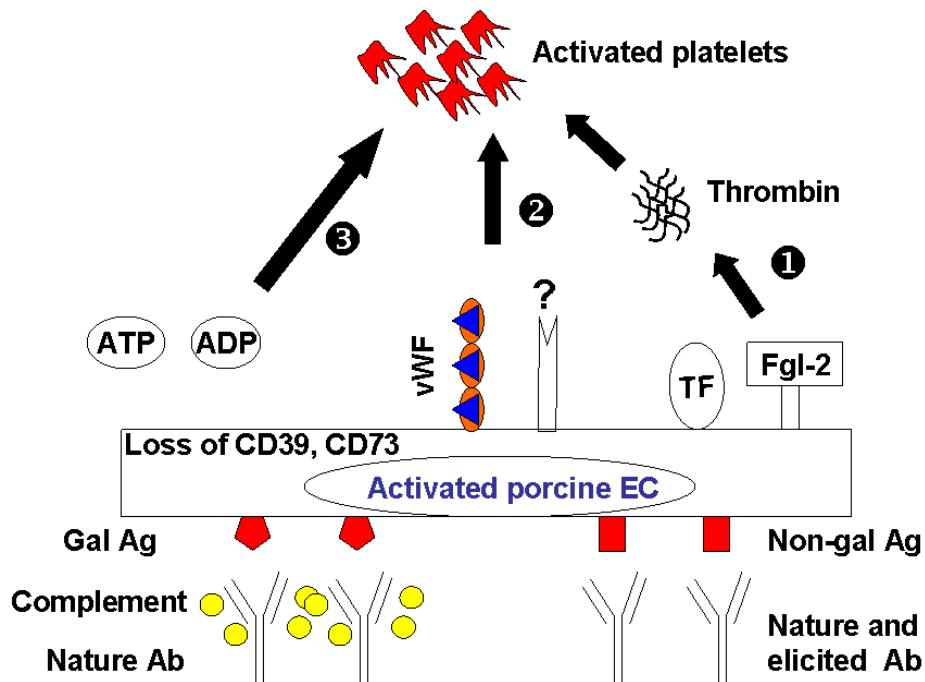


Figure 8.1-1 Diagrammatic representation of procoagulant change of PAECs and activation of platelets in pig-to-primate organ xenotransplantation.

(1) PAECs are activated by XNA with/without the involvement of complement to express TF and trigger the formation of thrombin, which is a potent activator of platelets. Fgl-2 cleaves prothrombin to generate the formation of thrombin. (2) The ligands (vWF or others) on the PAECs activate platelet receptors probably independent of the humoral immune response. (3) PAECs lose expression of CD39 and CD73, and are unable to adequately catalyse ADP to adenosine, and therefore contribute to the activation of platelets.

8.2 NON-IMMUNOLOGIC MECHANISMS CONTRIBUTING TO COAGULOPATHY DURING XENOTRANSPLANTATION

However, immune-independent factors, such as molecular incompatibilities, also contribute to the activation of platelets directly. The effect of porcine vWF has been mentioned already. In the present study, primate platelets and monocytes were activated to express TF when incubated with PAECs without the involvement of Abs and complement. This kind of activation of platelets and monocytes was confirmed in animal models of pig-to-primate kidney or liver xenotransplantation. In the kidney xenotransplantation model, baboon platelets and monocytes were activated to express TF after organ transplantation from GT-KO with/without MCP (+/+) pigs. The expression of TF on platelets and leucocytes was associated with the development of a consumptive coagulopathy. Importantly, although most recipients developed a severe systemic consumptive coagulopathy, the grafts were still functioning. Histological examination of grafts showed minimal features of immune injury, rather than AHXR or TM. In the liver xenotransplantation model, we clarified that the aggregation of platelets and formation of fibrin occurred even when the expression of VCAM-1 and TF on ECs in the grafts remained minimal. This dissociation suggests that this activation of platelets seems is unrelated immune response and is compatible with my *in vitro* findings. Once activated, platelets contain many pro-inflammatory mediators and surface ligands that are expressed and/or released. Importantly, activated platelets bind avidly to leucocytes, forming platelet-leucocyte complexes and inducing TF expression on leucocytes through P-selectin and its ligand on the leucocyte. TF expression on PBMCs continues to the

development of a systemic consumptive coagulopathy, which leads to irreversible graft failure.

8.3 INTERACTIONS BETWEEN PRIMATE PLATELETS AND PAECs

Since TF expression on platelets predicts the development of consumptive coagulopathy, the molecular interactions between primate platelets and PAECs will play a crucial role in resolving challenge. Compatible with other groups' studies, we demonstrated that PAECs activated primate platelets through the interaction of vWF on ECs and GPIb on platelets. Interestingly, this activation of platelets is dependent of the interaction of P-selectin and PSLG-1 on platelets. Additionally, platelet TF expression was inhibited by anti-platelet agents, such as eptifibatide and clopidogrel.

8.4 CONCLUSIONS

The present study demonstrated that TF expression on recipient platelets and PBMCs plays an essential role in consumptive coagulopathy. The effects result from non-immunological factors and lead to systemic complications. In contrast to the previous concepts, further manipulation of immune system and genetic modification of donor organs may not be sufficient to overcome this problem. Multiple modalities addressing different aspects of the problem are likely to be necessary for further advances.

8.5 FUTURE STUDIES TOWARDS TO SUCCESSFUL XENOTRANSPLANTATION

The resolution of this coagulation disorder is critical to allow xenotransplantation to advance sufficiently to clinical trials. Current studies have revealed that the coagulation disorder involves not only activation in the graft, which may be related to AHXR, but also systemic changes that take place in the circulation; these may be initiated by factors unrelated to the immune response. A clearer understanding of the molecular interactions between PAECS and primate platelets may provide new targets for intervention. Ideally, genetic modification of the organ-source pig offers the optimal approach to avoid adverse systemic effects. The generation of GT-KO pigs transgenic for one or more ‘anticoagulant’ or ‘anti-thrombotic’ genes is likely to confer additional benefit. However, the documented systemic coagulation disorders suggest the necessity of an additional pharmacological approach. We therefore propose that, in addition to genetic modification of the organ-source pig, systemic medication will be necessary. Hopefully, this combined approach will enable the adverse effects of coagulation disorders to be overcome.

8.5.1 Multiple –gene transgenic pigs

Although the introduction of GT-KO pigs as a source of organs has provided a significant advance, many unresolved problems have stimulated the necessity for additional genetic modifications of the organ-source pig. GT-KO pigs that express a hCRP and one or more ‘anticoagulant’ or ‘anti-thrombotic’ genes are anticipated to inhibit the generation of thrombosis and subsequent platelet activation. With new cloning techniques, transgenic mice for CD55/hTFPI/hTM/CTLA4-Ig/hCD39 have been attempted (Fisicaro et al.,

2007). The same technology is expected to be successfully applied to generate pigs with multiple gene modifications and will enable these pigs to become available in a shorter period of time than through conventional breeding.

8.5.2 Systemic pharmaceutical approach

The present study has demonstrated that atorvastatin is able to inhibit TF expression on PAECs activated by either primate serum or by complement-independent anti-nonGal Abs. Atorvastatin was also capable of preventing TF expression and subsequent platelet-leucocyte aggregates after pre-incubation with PAECs. Furthermore, lovastatin enhanced ecto-NAPDases activity and membrane expression in ECs (Ledoux et al., 2002), consequently inhibiting platelet aggregation through the action of adenosine, which plays an important role in platelet aggregation during AHXR. These promising *in vitro* results encourage us to investigate the effects of statins in pig-to-primate transplantation models.

Anti-platelet agents have been extensively used clinically to prevent or reduce the development of cardiovascular disease (Chan et al., 2003; Gurbel et al., 2005). Anti-platelet agents, such as antagonists of P2Y₁₂ or GPIIb/IIIa receptors, would be expected to prevent aggregation when platelets are activated during AHXR. We also demonstrated that eptifibatide and clopidogrel inhibit platelet TF expression after incubation with PAECs. The failure of anti-platelet drugs in previous studies may be because the immune response was not well-controlled at that time and its intensity was so strong to overshadow the benefit of anti-platelet agents. In the combination with genetic

modification of pig donors, thrombosis in the graft may be prevented by anti-platelet agents, which may offer additional benefits by the prevention of systemic coagulopathy.

APPENDIXES

Appendix 1: Protocol for PAECs culture medium

The stocking solution was prepared after sterilization using a filter system (Corning) and Stored at 4°C.

Reagent	Company	Volume
RPMI 1640	Gibco	450ml
Anti-mycotic liquid (penicillin, streptomycin, amphotericin B)	Invitrogen	5ml
HEPES buffer solution	Gibco	5ml
Fetal bovine serum (HI)	Sigma	50ml
Endothelial cell growth supplement	BD Biosciences	

Appendix 2 Protocol for PAECs washing medium

The stocking solution was prepared after sterilization using a filter system and stored at 4°C.

Reagent	Company	Volume
RPMI 1640	Gibco	450ml
Penicillin+ streptomycin	Invitrogen	5ml
HEPES buffer solution	Gibco	5ml
Bovine serum (HI)	Sigma	50ml

Appendix 3 Protocol for 0.05% collagenase solution

Reagent	Company	Volume
Collagenase B	Sigma	10mg
PBS	Invitrogen	20ml

Appendix 4 protocol for PAECs storage medium

The stocking solution was prepared after sterilization using a filter system and stored at 4°C.

Reagent	Company	Volume
RPMI 1640	Gibco	400ml
Fetal bovine serum (HI)	Sigma	100ml

Appendix 5: Protocol for MACS buffer

The stocking solution was stored at 4°C

Reagent	Company	Volume
PBS	Invitrogen	500ml
0.5 M EDTA	Sigma	2 ml
Fetal bovine serum	Sigma	2.5 gm

Appendix 6: Protocol for FACS solution

The stocking solution was stored at 4°C

Reagent	Company	Volume
PBS	Invitrogen	1000ml
Sodium Azide	Sigma	1 gm
Bovine serum albumin	Sigma	1gm

Appendix 7: Protocol for Tri base solution (TBS, 25 mM)

The stocking solution was autoclaved and stored at room temperature

Reagent	Company	Volume
Tris	Sigma	8 mg
NaCl	Sigma	3 gm
KCl ₂	Sigma	0.2 mg
Milli Q water		1000ml

Appendix 8: Protocol for ACK lysis buffer

The stocking solution was sterilized by 0.22µm filter and stored at room temperature.

Reagent	Company	Volume
NH ₄ Cl	Sigma	4.1 gm
KHCO ₃	Sigma	0.5 gm
EDTA-Na ₂	Sigma	18.6 gm
Milli Q water		500ml

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