

Pre-treatment of porcine pulmonary xenograft with desmopressin: a novel strategy to attenuate platelet activation and systemic intravascular coagulation in an ex-vivo model of swine-to-human pulmonary xenotransplantation

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Abstract: Background: Von Willebrand factor (vWF) has been proposed as a major contributor to the development of coagulopathy in pulmonary xenotransplantation. Pretreatment of donor swine with 1-deamino-8-D-arginine vasopressin (DDAVP), an analog of vasopressin, can reduce the content of vWF in pulmonary xenografts. Here, we investigate the effects of DDAVP pre-treatment in an ex-vivo perfusion model of pulmonary xenotransplantation.

Methods: We set up and performed the ex-vivo perfusion using porcine pulmonary accessory lobes and fresh human whole blood (n = 12).

Half of the donor swine were given 3 µg/kg DDAVP intravenously for 3 days before ex-vivo perfusion (DDAVP group) and half of them were left untreated (control group). The porcine lung was perfused with fresh blood for 1 h and changes in the following parameters were monitored: pulmonary arterial pressure, pulmonary vascular resistance, blood cell counts, fibrinogen, antithrombin, platelet factor 4, D-dimer, C3a, C4d, and xenoreactive IgM. The release of Gal α 1-3Gal xenoantigen (α Gal) from porcine lung which had been perfused and retained for 30 min with human blood was assessed by enzyme-linked immunosorbent assay using α Gal-binding lectin.

Results: Both DDAVP and control groups showed typical findings of immediate pulmonary dysfunction: an increase of pulmonary vascular resistance and sequestration of leukocytes and platelets after ex-vivo perfusion. However, in the DDAVP group, the increase of platelet factor 4, C3a, and C4d after perfusion was attenuated compared to that in the control group. The release of α Gal after blood retention was significantly lower in the DDAVP group than that of the control group.

Conclusion: Pre-infusion of DDAVP to the donor swine was beneficial in attenuating platelet activation as well as complement/coagulation activation. These effects of DDAVP are likely to relate to the reduction of porcine vWF content in the xenograft. Therefore, the modulation of vWF secretion in donor lungs could be an additional therapeutic way to reduce systemic coagulopathy in pulmonary xenotransplantation.

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Key words: complement – 1-deamino-8-d-arginine vasopressin – lung – platelet – von Willebrand factor – xenotransplantation

Abbreviations: AU, artificial unit; DDAVP, 1-deamino-8-d-arginine vasopressin; ELISA, enzyme-linked immunosorbent assay; α Gal, Gal α 1-3 Gal xenoantigen; GSL, *Griffonia simplicifolia* lectin; PAEC, porcine aortic endothelial cells; PBS, phosphate-buffered saline; PF4, platelet factor 4; PG, prostaglandin; PVR, pulmonary vascular resistance; TBS, Tris-buffered saline; vWF, von Willebrand factor.

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Introduction

Xenotransplantation using porcine organs is a promising alternative solution to overcome the shortage of human donor organs. Hyperacute rejection, the first hurdle for xenotransplantation, could be overcome using porcine organs genetically engineered to overexpress human complement regulatory proteins or to lack the major xenoantigen, Gal α 1-3Gal (α Gal) [1]. These strategies enabled us to prolong the survival of porcine xenografts transplanted to non-human primates up to several months or weeks in cardiac and renal transplantation [2–5]. However, in the case of pulmonary xenotransplantation, xenografts undergo a rapid failure immediately after exposure to human or primate blood [6–8], characterized by elevated pulmonary vascular resistance (PVR) and pulmonary edema associated with vascular thrombosis [7], despite these strategies. Intravascular thrombosis accompanying systemic coagulopathy is a main pathologic feature of pulmonary xenografts while tissue necrosis and hemorrhages are the main attributes of cardiac or renal xenograft hyperacute rejection [9].

Von Willebrand factor (vWF) has been proposed as a major contributor to the development of coagulopathy in pulmonary xenotransplantation [9,10]. Porcine vWF released from activated endothelial cells of xenografts not only promotes platelet adhesion and activation on the surface of endothelial cells, but also provides binding sites for xenoreactive antibodies because of its abundant α Gal moiety. The immune complexes of xenoreactive antibodies and vWF on swine endothelial cells are shed into the circulation and lead to systemic coagulation abnormality [11]. We have previously demonstrated that pre-treatment of donor swine with 1-deamino-8-D-arginine vasopressin (DDAVP), an analog of vasopressin, can reduce the content of vWF in pulmonary xenografts and ameliorate platelet sequestration in the swine-to-canine pulmonary xenograft model [12]. Accordingly, we asked if DDAVP pre-treatment would be beneficial in swine-to-human xenotransplantation. Consequently, we investigated the effect of DDAVP pre-treatment of donor swine in an ex-vivo perfusion pulmonary xenotransplantation model.

Materials and methods

Treatment of donor with DDAVP

All of the animals in the study were treated humanely in compliance with the “Guide for the Care and Use of Laboratory Animals” published

by the National Institutes of Health (NIH Publication No. 86–23, revised 1985). DDAVP treatment was performed as previously described [12]. Briefly, six adult donor swine (22 to 35 kg) were anesthetized with intramuscular administrations of ketamine hydrochloride (20 mg/kg) and were intubated and ventilated with 100% oxygen at a rate of 10 times/min with a tidal volume of 12 ml/kg. A 9 Fr. Hickman catheter (Bard Access Systems, Salt Lake City, UT, USA) was inserted into the external jugular vein through the neck incision and the tip was placed in the back. DDAVP (3 μ g/kg; Ferring Pharmaceuticals Inc., Suffern, NY, USA) was injected every 12 h intravenously through the Hickman catheter for 3 days until experiment of ex-vivo perfusion. Blood was sampled at the time of insertion of the Hickman catheter and at the time of organ harvest.

Ex-vivo perfusion set up

The human blood for perfusion was donated from healthy volunteers with B+ blood type. The blood donation for research purposes was approved by the Institutional Review Board (IRB H-0412-138-013). Four hundred microliters of blood was drawn in a blood bag containing the anticoagulant citrate-phosphate-dextrose-adenine at 2 to 3 h before the ex-vivo perfusion. The blood was diluted with the same volume of Hartmann’s solution and was added with 2 units/ml of heparin sodium and 1.3 mg/ml of CaCl₂. The perfusate was primed in the ex-vivo perfusion circuit.

Lung harvest

Six swine for each DDAVP-infused and control group were used for the experiment. Each animal was anesthetized by intramuscular injection of ketamine hydrochloride (20 mg/kg), xylazine (1 mg/kg), and atropine (0.2 mg). After endotracheal intubation, general anesthesia was maintained with sevofluene gas inhalation and intermittent vecuronium administration. The animal was ventilated with a tidal volume of 15 ml/kg and respiration rates of 15 times/min. A right thoracotomy was placed and the pleural cavity was entered through the top of 7th rib. The accessory lobe was identified by opening of the pleural investment and the pulmonary artery, vein, and bronchus were dissected. The fissure was carefully divided, avoiding parenchymal damage. The animal was heparinized (300 units/kg) and the accessory lobe was harvested. We divided the pulmonary artery before division of the pulmonary vein to avoid any chance of congestion of the graft.

After that, the bronchus was divided. The resected accessory lobe was placed in cold preservation solution and the pulmonary artery was cannulated with 8 Fr. aortic cannula, through which prostaglandin (PG) E1 (Eglandin; 4 µg/kg, Welfide, Hwaseong, Korea) was infused. The accessory lobe was then perfused with 4 °C modified Euro-Collins preservation solution (100 ml/kg). During the perfusion, the accessory lung was ventilated through the bronchial cannula with room air. After perfusion, the pulmonary vein was cannulated for ex-vivo perfusion. A portion of the remaining right upper lobe was sampled for tissue control. The left lung was harvested for the perfusate retaining model.

Ex-vivo perfusion model

The accessory lobe was connected to the ex-vivo circuit. The perfusion was slowly started and the flow rate was increased gradually to reach 20 ml/min at the pulmonary venous drain site. The graft was ventilated with room-air at 20 ml of tidal volume and 20 times/min of respiratory frequency using a Harvard rabbit ventilator (Hugo Sachs Elektronik, March Hugstetten, Germany). The temperature was maintained at 37 °C with a heat-exchanger and water bath. The perfusion pressure was monitored and blood was sampled at both pulmonary arterial and venous sides at 10, 30, and 60 min after perfusion. After 60 min of perfusion, the graft tissue was obtained.

Perfusate retaining model

The left pulmonary artery was cannulated with a 24 Fr. aortic cannula and was perfused with PGE1 and cold Euro-Collins solution. The 200 ml of perfusate (1 : 1 mixture of human blood and Hartmann's solution) was infused through the cannula at 40 cm height and the pulmonary vein was clamped. After 30 min of retention, the pulmonary venous clamp was released and the retained blood was drawn and sampled.

Measurement of blood cell counts and coagulation/complement activation markers

We monitored changes in the following parameters up to 60 min after reperfusion: blood cell counts, and plasma levels of fibrinogen, antithrombin III, D-dimer, platelet factor 4 (PF4), C3a, and C4d. Blood cell count was measured by ABCvet CBC analyzerTM (ABX hematology, Montpellier Cedex, France) and coagulation parameters including fibrinogen, antithrombin III, and D-dimer by STA Compact analyzerTM (Diagnostica Stago,

Asnières, France). PF4 was assayed by enzyme-linked immunosorbent assay (ELISA) using the Asserachrom[®] PF4 kit (Diagnostica Stago). The complement activation fragments C3a and C4d were also measured by ELISA kits (Quidel, San Diego, CA, USA). Each parameter was expressed as a percentage of baseline value prior to reperfusion.

Assessment of circulating xenoreactive IgM and IgG

Flow cytometric analysis was performed to detect IgM and IgG xenoreactive antibodies. Porcine aortic endothelial cells (PAEC) were purchased from Cell Applications (San Diego, CA, USA). Briefly, 5×10^5 PAEC were incubated with 50 µl of plasma in each tube for 30 min at 37 °C. In parallel, the cells were incubated with phosphate-buffered saline (PBS) as a negative control, and with a serial dilution of the selected human plasma as a calibrator, which was confirmed to have high titer of IgM antibodies against PAEC. The cells were washed with PBS and then incubated with 100 µl of 1 : 200 diluted rabbit anti-human IgM (Dako Corp., Carpinteria, CA, USA) or fluorescein isothiocyanate (FITC)-labeled rabbit anti-human IgG (Dako Corp.) for 30 min at 37 °C. The cells incubated with unlabeled anti-human IgM were further stained with 1 : 200 diluted FITC-labeled anti-rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, OH, USA) for 30 min at 37 °C in the dark. After washing, the cells were fixed with 500 µl 1% paraformaldehyde and then analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). The geometric mean fluorescent intensity of each sample was compared to those of the negative control and the calibrator. The amount of binding antibody was expressed as an artificial unit (AU)/ml compared to the mean fluorescent intensity of undiluted calibrator designated as 100 AU/ml.

Measurement of αGal level in perfusate

The level of αGal in perfusate was determined by ELISA using a *Griffonia simplicifolia* lectin (GSL) I-isolectin B₄ (Vector laboratories, Burlingame, ON, Canada) coated plate and biotinylated GSL I-isolectin B₄ (Vector laboratories). Briefly, the microwell plate was coated with 10 µg/ml GSL I-isolectin B₄ in carbonate buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6) overnight at 4 °C. The wells were blocked with Tris-buffered saline (TBS), pH 8.0 containing 1% bovine serum albumin for 1 h. Each well was reacted with 100 µl of 1/10 diluted serum in TBS for 30 min at 37 °C. The

pooled normal swine serum serially diluted in normal human serum was tested in parallel and used as a control. After being washed with TBS containing 0.05% Tween 20, the wells were reacted with 100 μ l of 1 μ g/ml biotinylated GSL I-isolectin B₄ for 30 min at 37 °C and then 100 μ l of 1 : 2000 diluted horseradish peroxidase-conjugated streptavidin (Dako Corp.) for 30 min at 37 °C. After washing, the color reaction was developed and the absorbance at 450 nm was measured.

Statistical analysis

Data are expressed as mean \pm standard error of the mean. Comparisons between groups were analyzed using a mixed model which is a type of repeated measures analysis but applicable to analysis of unbalanced data. A P-value less than 0.05 was considered significant.

Results

Hemodynamic parameters

The pulmonary arterial pressure increased in both control and DDAVP groups after perfusion with human blood ($P = 0.001$). Initially, the mean pulmonary arterial pressure was 37.0 ± 18.0 mmHg and 46.3 ± 14.6 mmHg in the control and DDAVP groups, respectively. After 60 min perfusion, it increased to 57.5 ± 7.3 mmHg and 68.0 ± 1.0 mmHg in control and DDAVP groups, respectively. Compared to the control group, the DDAVP group showed higher pulmonary arterial pressure ($P = 0.022$). In the control group, pulmonary vascular resistance increased from 1.8 ± 0.6 mmHg/ml/min to 3.0 ± 0.5 mmHg/ml/min, and in DDAVP group, from 2.2 ± 1.4 mmHg/ml/min to 3.0 ± 0.1 mmHg/ml/min. There was, however, no statistically significant difference between the two groups ($P = 0.635$).

DDAVP pre-treatment prevents subsequent hemoconcentration after perfusion.

We monitored blood cell counts in the perfusate prior to and during the perfusion. The initial blood counts were not different between groups. Immediately after the start of perfusion, platelet counts in both groups rapidly decreased within 10 min and remained low throughout the experiment (Fig. 1). Leukocyte counts gradually decreased with time and there was no difference between groups. Hemoglobin levels in the whole blood gradually increased during the experiment in both

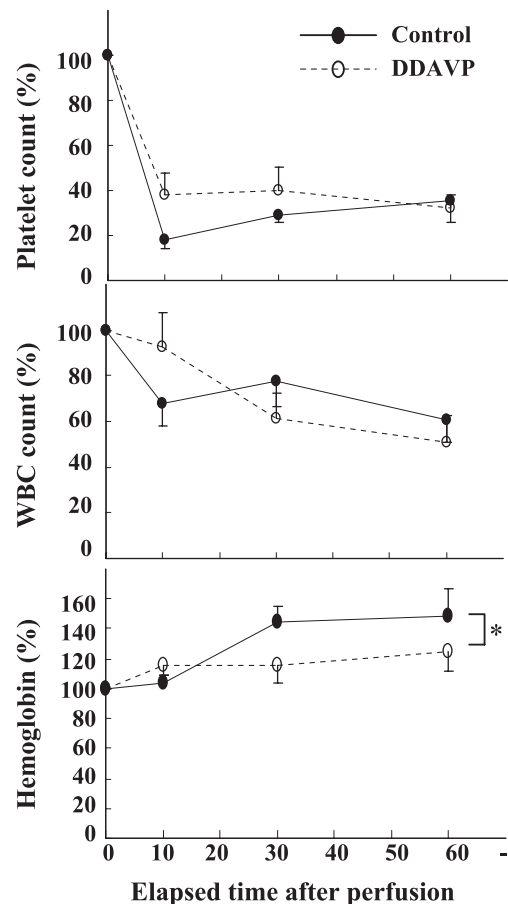


Fig. 1. Changes in hematological parameters: leukocyte and platelet counts and hemoglobin level during ex-vivo perfusion. Half of donor swine were given 1-deamino-8-D-arginine vasopressin (DDAVP, 3 μ g/kg) intravenously every 12 h for 3 days before ex-vivo perfusion ($n = 6$ for each group). The porcine lung was perfused with fresh human whole blood and its hematological parameters were monitored for 60 min. Each value at a given time point is expressed as the percentage of the initial measurement. Increases in hemoglobin levels were higher in the control group than that of the DDAVP group (* $P = 0.032$).

groups but the increase in the control group was higher than that of the DDAVP group ($P = 0.032$), suggesting that hemoconcentration developing during the perfusion was severer in the control group than in the DDAVP group.

DDAVP pre-treatment attenuates systemic coagulation abnormalities and platelet activation

We timely measured the fibrinogen, antithrombin III, and D-dimer levels in perfusate to monitor the coagulation system. Fibrinogen and antithrombin III levels did not change significantly during the perfusion and were not different between groups (Fig. 2). The D-dimer level in the control group occasionally increased during the perfusion and

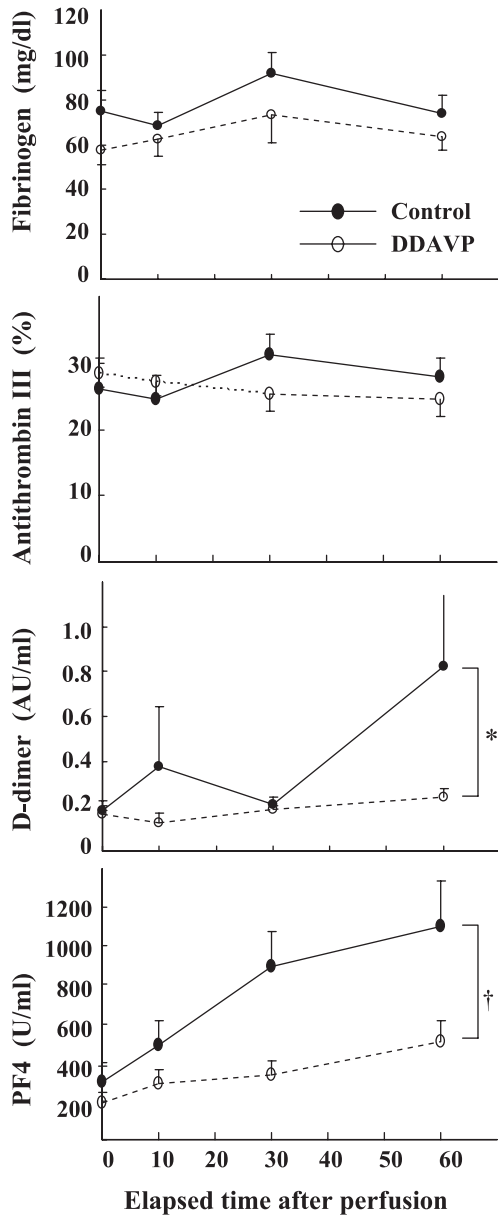


Fig. 2. Changes in coagulation parameters: fibrinogen, anti-thrombin III, platelet factor 4 (PF4), and D-dimer during ex-vivo perfusion. Porcine lungs of both DDAVP pretreated and control groups were perfused with fresh human blood for 60 min and blood was sampled at the given time points. Four coagulation parameters were assayed. In the DDAVP group, D-dimer levels tended to be lower than those of the control group and PF4 levels were significantly lower (*P = 0.138; †P < 0.001).

tended to be higher than that of the DDAVP group (P = 0.138). In the DDAVP group, the level of D-dimer remained in the normal range throughout the duration of perfusion.

The level of PF4 was also measured to monitor platelet activation. It increased steadily during perfusion in both groups. However, the increase was significantly less in the DDAVP group than

that of the control group (P < 0.001, Fig. 2), demonstrating that the extent of platelet activation was less in the DDAVP group compared to controls.

DDAVP pre-treatment reduces complement activation in ex-vivo perfusion model

To evaluate activation of the complement system, C3a and C4d were monitored during perfusion. In the control group, C3a and C4d levels increased within 10 min after perfusion and were maintained at high concentrations throughout the experiment (Fig. 3). However, the DDAVP group showed only a small increase in C3a and C4d levels during the perfusion and their C3a and C4d levels tended to be lower than those of the control group (P = 0.057, 0.058), indicating that less systemic complement activation occurred in the DDAVP group.

αGal release from xenograft diminishes in DDAVP pre-treated group

In the next step, we asked how DDAVP pre-treatment can reduce complement activation. Porcine vWF is known to carry a lot of αGal moiety,

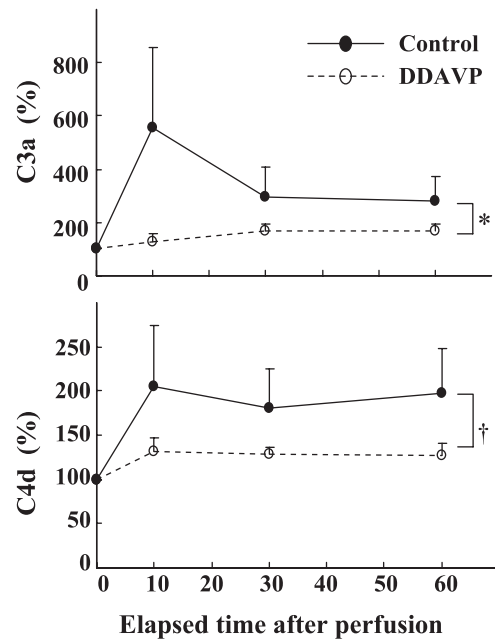


Fig. 3. Changes in complement activation products: C3a and C4d during ex-vivo perfusion. Each DDAVP pretreated and control porcine lung was perfused with fresh human blood for 60 min and C3a and C4d in the plasma were monitored. C3a and C4d levels increased within 10 min after perfusion but the increase of each during perfusion tended to be lower in the DDAVP group than in the control group (*P = 0.057; †P = 0.058).

which is a main xenoantigen. We hypothesized that depletion of vWF by DDAVP pre-treatment would reduce the amount of vWF released into the circulation, which would lead to the subsequent reduction of complement activation and systemic coagulation. To determine the amount of released α Gal antigen, we tried to detect α Gal by ELISA using GSL I-isolectin B₄ in the perfusate timely drawn during the ex-vivo perfusion. However, we failed to demonstrate the presence of α Gal in the perfusate of either group (data not shown). We designed a perfusate retaining model with the expectation of exaggerated responses of endothelial cells. Each xenograft was perfused and retained for 30 min without circulation, and the perfusate was obtained thereafter. α Gal was easily detectable by ELISA and its level was higher in the control group than in the DDAVP group ($P = 0.0317$, Fig. 4), demonstrating that DDAVP pre-treatment reduces the release of α Gal from xenograft into the circulation.

The initial level of IgM anti-swine antibodies in the perfusate was not different between groups

Complement activation in xenografts is known to depend on the level of anti-swine antibody in circulating blood. The less complement activation in the DDAVP group might have been caused by low anti-swine antibody levels of each blood donor. To exclude this possibility, we measured the level of anti-PAEC antibodies in the blood by flow cytometry. The blood used in each experiment showed a variable level of IgM antibodies against PAEC, but on average, the initial level of IgM anti-

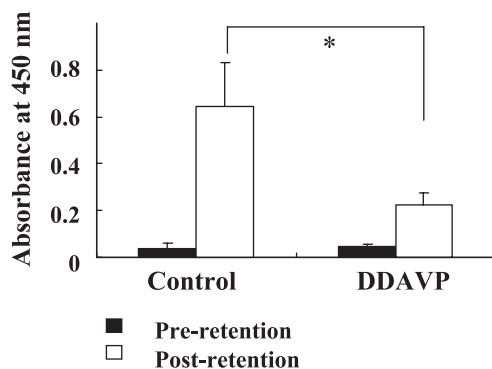


Fig. 4. α Gal release from DDAVP treated and control porcine lungs. Each lung was perfused with fresh human blood and retained for 30 min ($n = 6$ for each group). The blood was obtained before and after 30 min retention. Serum α Gal was captured on GSL I-isolectin B₄ coated wells and detected by biotinylated GSL I-isolectin B₄ and subsequent color development. α Gal level was higher in the control group than in the DDAVP group (* $P = 0.0317$).

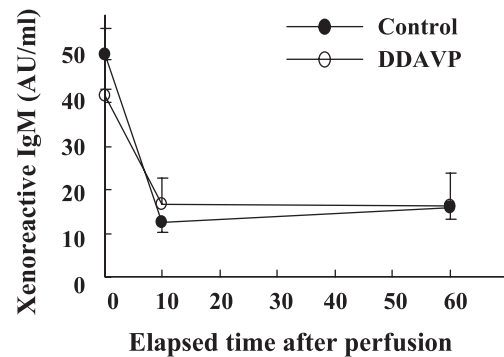


Fig. 5. Change of xenoreactive IgM antibody titer during ex-vivo perfusion. Each lung of DDAVP pretreated or control swine was perfused with fresh human blood for 60 min and blood was obtained at given time points. Porcine aortic endothelial cells were incubated with each serum, sequentially reacted with rabbit anti-human IgM and FITC-labeled anti-rabbit IgG, and analyzed with a flow cytometer. The titer of xenoreactive IgM antibody was determined by the mean fluorescent intensity of each serum and expressed as an artificial unit (AU)/ml compared to the mean fluorescent intensity of undiluted calibrator designated as 100 AU/ml. There was no significant difference in the initial titer and overall change of xenoreactive IgM between DDAVP treated and control groups.

PAEC antibodies in the perfusate was not different between groups (Fig. 5). The level of IgM anti-PAEC antibodies rapidly decreased after perfusion in both groups suggesting xenoreactive IgM antibodies are captured and sequestered in the xenograft. We tried to measure IgG antibodies against PAEC but failed to detect any significant binding of IgG antibodies to cells (data not shown).

Discussion

In this study, we demonstrated that DDAVP pre-treatment of donor swine reduces adverse responses related to platelet activation and systemic coagulation/complement activation in an ex-vivo perfusion model of pulmonary xenotransplantation. When porcine lung was perfused with fresh human blood, blood platelet counts rapidly dropped and the levels of D-dimer, PF4, and complement activation fragments increased, suggesting that platelets were activated and sequestered into the xenograft, subsequently resulting in intravascular coagulation and complement activation. However, in DDAVP pre-treated group, the degree of increase for D-dimer, PF4, C3a, and C4d after ex-vivo perfusion was attenuated and their post-perfusion levels were significantly lower than those of the control group.

The DDAVP is known to induce vWF secretion from endothelial cells [13]. Porcine vWF is considered a culprit for intravascular thrombosis and

systemic coagulation in pulmonary xenotransplantation [9,11]. Following exposure to human blood, porcine endothelial cells are activated possibly by multiple stimuli, such as ischemic or mechanical injury, and binding of xenoreactive antibodies with or without subsequent complement activation, leading to exposure of pro-coagulant and pro-inflammatory surfaces and expression or secretion of P-selectin, vWF, and platelet-activating factor [14–16]. Porcine vWF binds to human platelets and induces their activation and aggregation [17]. A study of swine-to-baboon pulmonary xenotransplantation using vWF-deficient donors has shown that vWF-deficient xenografts exhibit interstitial hemorrhage and edema instead of intravascular thrombosis, one of the typical features of pulmonary xenotransplantation rejection [9]. This suggests that porcine vWF is the key molecule for the development of intravascular thrombosis in pulmonary xenografts. In previous study, we demonstrated that pre-treatment of donor swine with DDAVP reduces the content of vWF in swine lung without up-regulation of vWF synthesis [12]. The DDAVP treatment of donor swine reduced the content of vWF Ag in porcine lung tissue by 52% (DDAVP-treated group vs. control group, 7.7 ± 2.4 AU/mg vs. 16.0 ± 5.6 AU/mg). We speculate that about 50% of vWF would be left in porcine lung tissue after treatment even though the exact distribution and functional impact is unknown. We have previously shown that DDAVP pre-treatment of donor swine attenuates the decrease of blood platelet counts in swine-to-canine pulmonary xenotransplantation [12]. In this study, DDAVP pre-treatment could not prevent the decrease of platelet counts in human blood after ex-vivo perfusion. However, it certainly reduced the increase of PF4 which is a platelet activation marker. D-dimer, a marker of systemic intravascular coagulation, remained in the normal range in the perfusion of DDAVP pre-treated porcine lungs, while it frequently increased in that of control lungs. These beneficial effects of DDAVP which were shown not only in the contact with canine blood but also in that with human blood can be attributable to the possible reduction of vWF content in xenografts by DDAVP treatment.

Porcine vWF is also known to contain α Gal, a major antigen for xenoreactive antibodies [18]. When activated, porcine pulmonary endothelial cells shed vWF along with other membrane proteins [11], which forms circulating immune complexes with xenoreactive antibodies, leading to type III immune complex disease and disseminated intravascular coagulation [11]. To further explore

the DDAVP effect on the formation of immune complexes, we first tried to demonstrate the presence of circulating porcine-vWF/IgM immune complex in blood by ELISA but failed to get a positive signal. Next, we tried to measure the amount of α Gal released into circulating blood by ELISA using GSL I-isolectin B₄. Disappointingly, α Gal was not detected in blood samples from the ex-vivo perfusion model. However, the signal in the positive control well tested in parallel was clearly positive. We assumed that the concentration of released α Gal was too low to be detected by our ELISA system. Consequently, we designed a perfusate retaining model to concentrate α Gal in the blood. Soluble α Gal was not detected in blood samples obtained before retention but it was obviously detectable by ELISA in samples obtained after retention. Its concentration was significantly higher in the control group than in the DDAVP group. This finding demonstrates that DDAVP pre-treatment reduces the release of α Gal from xenografts into the circulation. We speculate that shed porcine vWF would be an important source of circulating α Gal in xenotransplantation and partial depletion of vWF by DDAVP provides a benefit in the reduction of α Gal release and its immune complex formation, and in the prevention of subsequent complement and coagulation activation. Lower level of C3a and C4d in the perfusate of DDAVP group is consistent with our speculation. These findings support the implication of porcine vWF for intravascular thrombosis and systemic complement/coagulation abnormalities in pulmonary xenotransplantation.

Porcine α Gal epitope has the carbohydrate structure similar to blood type B, and anti-B antibodies in A and O individuals can recognize both epitopes of α Gal and blood type B [19]. Actually, the antibody to recognize both α Gal and blood type B comprises about 85% of natural anti-B antibodies in A and O individuals. Because we didn't have any attempt to inhibit xenoreactive antibodies and complement in this study, we were afraid of possible massive hyperacute rejection initiated by anti-B antibodies. Blood type AB human blood would be the best to avoid any effect of ABO mismatch but the low frequency of AB type in our population forced us to recruit donors with blood type B as a practical alternate. As for donor animal, we should have used blood type O swine, but in our situation, the pigs bred for experimental purpose were not available and we had to use farm pigs. In this process, the selection of certain blood type of swine was impossible for us. Accordingly, we cannot exclude the possibility that anti-A antibodies along with anti- α Gal

antibodies in perfusion blood could have played a role in injury of the lung in the case of blood type A swine.

For comparisons between groups, we used mixed model, a type of repeated measures analysis. Its advantage is applicability to the analysis of unbalanced data. The data of repeated measurements from a subject have autocorrelation. Analysis of longitudinal or repeated measures data for balanced study design can be done using repeated measures analysis of variance. However, balanced data sets are rarely obtained in studies and have caused serious statistical analysis problem. The basic theory on which mixed model are based holds with unbalanced data. In this study, we compared the trend of the data according to the elapsing time, not the data at a given time point. Accordingly, some data set at a given time looked inconsistent with the P-value which reflected significance of difference between groups for whole experimental duration.

Pulmonary vascular resistance (PVR) is an important variable of lung function but there was no difference in the PVR between groups in this study. This is not surprising given the fact that there was no attempt to inhibit antibodies or complement. However, in our perfusion setting, the graft should be perfused gently by increasing perfusion flow gradually at the beginning. As a consequence, PVR and other hemodynamic changes earlier than 5 to 10 min of perfusion were not completely evaluated. Therefore, there might be a more subtle effect on PVR early in the first minutes of perfusion, which we might have missed. Further improvement of ex-vivo perfusion setting is necessary to evaluate earlier events of perfusion.

In this study, we clearly demonstrated the beneficial effect of DDAVP pre-treatment in pulmonary xenotransplantation using an ex-vivo perfusion model despite no additional strategies against either xenoreactive antibodies or complement activation. Additionally, pre-treatment of donor swine would not impose any harm on the recipient. Although vWF depletion by DDAVP would have a temporary and limited effect in preventing pulmonary xenograft dysfunction, theoretically, the reduction of initial inflammation in the graft would be desirable to minimize acute or chronic rejection. So far, no single strategy has been successful in prolonging the survival of pulmonary xenotransplantation and multi-strategic approaches are needed. Therefore, modulation of vWF is expected to provide an additional benefit to pulmonary xenotransplantation using genetically modified swine lacking α Gal expression and/

or expressing human complement regulatory proteins.

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