In Vivo Regulation of von Willebrand Factor Synthesis
von Willebrand Factor Production in Endothelial Cells After Lung Transplantation Between Normal Pigs and von Willebrand Factor–Deficient Pigs

Jean-Philippe Brouland, Thomas Egan, Jacqueline Roussi, Michel Bonneau, Georges Pignaud, Claire Bal, Marcel Vaiman, Patrick André, Philippe Hervé, Guy Michel Mazmanian, Ludovic Drouet

Abstract—To evaluate the regulation of plasma von Willebrand factor (vWF) and its in situ production by endothelial cells (ECs), 12 swine leukocyte antigen (SLA)-compatible left lung transplantations were performed. Normal lungs were transplanted into 10 pigs homozygous for von Willebrand disease and into 2 normal pigs. Additionally, 1 normal pig underwent pneumonectomy, and 1 SLA-incompatible lung transplantation between normal pigs was performed. None of the transplanted animals received immunosuppressive therapy. Plasma vWF level was evaluated by ELISA and multimeric pattern. EC vWF content was assessed by immunohistochemistry. Global hemostasis was assessed by standardized ear bleeding time. Six of 12 SLA-compatible lung transplantations and the incompatible transplantation were successful and were used for the study. The functions and the viability of ECs, reflected by their ability to produce vWF and normal multimeric plasma vWF pattern, were preserved in SLA-compatible and -incompatible lung transplantations. vWF production was preserved in ECs that initially synthesized it. EC constitutive and storage pathways are modulated differently according to transplantation compatibility and severity of rejection. In SLA-compatible lung transplantations without histological evidence of rejection, the production of vWF was preserved, whereas constitutive vWF secretion appeared to be altered in cases with minor histological signs of rejection. In pigs with von Willebrand disease that were transplanted with normal lungs without sign of rejection, plasma vWF was significantly increased in an amount expected from the estimated production of a normal lung. In the transplanted normal lung, there was no vWF overexpression by the ECs and no recruitment of ECs that initially did not express vWF. In SLA-incompatible transplantation, ECs were morphologically normal with increased and blurred vWF labeling, whereas plasma vWF levels remained normal, reflecting that EC activation is associated with an increased vWF production with probable diversion to storage pathway. This model depicts the changes of EC regulation of vWF secretion in pig lung transplants. However, this model cannot be directly extrapolated to human organ transplantation because animals did not receive any immunosuppressive therapy, which may be toxic to ECs. (Arterioscler Thromb Vasc Biol. 1999;19:3055-3062.)

Key Words: endothelial cells ■ lung transplantation ■ von Willebrand factor ■ immunohistochemistry ■ pigs

Endothelial cells (ECs), located at the interface between the blood stream and blood vessel wall,1 are involved in various physiological and pathological processes, notably graft rejection. The functional modulation of engrafted ECs has been well documented in terms of immunologic response parameters, such as expression of adhesion molecules and cytokines.2–4 However, there is little information about the modulation of products that are constitutively expressed by ECs and that may reflect their functional state after transplantation. Among the numerous products expressed by ECs that may be appropriate for study, von Willebrand factor (vWF) is one specific EC marker.5–8 vWF is a large multimeric glycoprotein synthesized by ECs and megakaryocytes.9 It is present in blood—both in plasma and inside platelets—and in ECs and the matrix of the blood vessel wall. vWF is synthesized as a large pro-vWF that dimerizes in the endoplasmic reticulum and multimerizes in the Golgi apparatus, in which acidic pH conditions10 necessary for the multimerization process are present.11 In ECs, the largest vWF multimers are stored in endothelium-specific organelles called Weibel-Palade (WP) bodies.12,13 vWF can be released through 2 different pathways: (1) a constitutive secretory pathway where vWF is liberated from

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3055
both cellular sides, luminal and abluminal, in the plasma and subendothelial matrix and (2) a storage pool, with inducible release, where the highest molecular forms of vWF are stored in the WP bodies. This adhesive glycoprotein, of nonhepatic origin, is involved in platelet adhesion to connective tissues and exposed subendothelium14–24 and in platelet aggregation.25,26 In the plasma, it binds and protects coagulation factor VIII from proteolytic degradation.27

Although plasma vWF is a widely accepted marker of endothelial activation, there are still few studies describing the regulation of vWF expression by ECs in pathological disorders.5,28,29 In nonpathological states, the regulation of EC vWF expression has been observed in pigs to be modulated by age and the location of the vascular bed.30–33

The regulation of vWF synthesis and release in the plasma is still incompletely understood. The basal plasma vWF variations, physiological and pathological, may result from changes in the constitutive pathway and/or stimulation of release from the regulated pathway. In vitro, vWF released from WP bodies is found after stimulation by various substances, such as thrombin,34 histamine,35 fibrin,36,37 and platelet-activating factor, and by complement components and oxidative stress.38 In vivo, in humans, plasma vWF level is increased in response to substances that raise cAMP levels, such as endotoxin,39 epinephrine,40 and desmopressin. This increase in plasma vWF is thought to be due to the release of vWF from WP bodies.

Pigs with von Willebrand disease (vWD) exhibit a recessive autosomal disease41,42 phenotypically identical to the human vWD type 3,3,43,44. The homozygous form is characterized by an increased bleeding time that is due to levels of plasma, platelet, endothelial, and subendothelial vWF lower than the limits of detection and the subsequent decrease of plasma levels of factor VIII.

The lungs are very rich in ECs, and pig lung ECs exhibit a striking and interesting heterogeneity of vWF expression.31 Thus, ECs from pulmonary artery and veins and from mucosal and peribronchial capillaries strongly express vWF, whereas ECs from alveolar capillaries, which represent the major mass of lung ECs, either do not express vWF or express vWF very weakly.31

This peculiar feature and the fact that the majority of plasma vWF originates from ECs45 prompted us to develop an original animal model based on various combinations of swine leukocyte antigen (SLA)-compatible single lung transplantations between normal and vWD pigs in order to study in vivo the synthesis and release of EC vWF.

Methods

Animals

Large White pigs with normal phenotype (NN, n = 17) or with homozygous vWD phenotype (WW, n = 10) came from the colony of the Institut National de la Recherche Agronomique (INRA, Rouillet, France [breeding facilities] and Jouy-en-Josas, France [experimental facilities]). The colony was established from the Mayo Clinic (Rochester, Minn) colony by collaboration with E.J.W. Bowie. Heterozygous vWD pigs were cross-bred with respect to the swine leukocyte alloantigens (SLA)46 in order to give birth to normal vWD pigs as recipients for left lung allotransplantations. The transplants were performed between SLA-identical pigs in 12 cases (Nos. 1 to 12) and between SLA-incompatible normal pigs in 1 case (No. 13) to assess the effects of complete SLA incompatibility on endothelium. Additionally, 1 normal pig underwent left pneumonectomy to evaluate the effect of pneumonectomy on plasma vWF level (No. 14).

Left Single Lung Allotransplantation

Surgery was performed in the Laboratoire de Chirurgie Expérimentale de Hôpital Marie-Lannelongue, Le Plessis Robinson, France. Transplantations were performed between pigs with similar weight to avoid difficulties resulting from lung size.

Anesthesia was performed as described previously47 to avoid any hematologic changes in pigs. Donors were intubated and ventilated. After sternotomy and opening of the pleura and pericardium, 400 mL of whole blood was retrieved for blood transfusion to recipients. The aorta was cross-clamped, and the lungs were flushed with 2 L cold modified Euro-Collins solution (70 mL of 50% dextrose and 8 Meq MgSO4/L) immediately after injection of 500 mg iloprost. The heart was excised, then both lungs were removed, and the left lung was stored inflated in cold modified Euro-Collins solution. Left lung transplantation was performed in recipients through a left thoracotomy by continuous suturing of the left pulmonary artery and left atrial cuff with the use of Mersilene (Davis & Geck) and interrupted suturing of the bronchus with 3–0 Maxon and Dexon II (Davis & Geck). Systemic bronchial artery flow was not restored.

Antibiotic (ampicillin, 0.1 g/kg for 24 hours) and analgesic (morphine) therapy was given for 5 days after surgery. In the immediate postoperative period, homozygous vWD pigs received 50 U/kg purified vWF concentrate (kindly provided by Dr. J. Savage from Porton Speywood, Ltd, Birmingham, UK) and red blood cells from SLA-compatible donors. No immunosuppressive therapy was instituted.

Technetium Scintigraphy

Exploration of the lung transplant circulation was performed by use of gamma scintigraphy (Maxicamera 2, General Electric, and Perilcolor 1000, Numelec) after intravenous infusion of technetium Tc 99m-labeled albumin microspheres (1 mCi/10 kg, International Cis).

vWF Antigen Determination

Plasma vWF antigen was assessed by ELISA53 using a monospecific rabbit anti-pig vWF antibody kindly provided by Dr Amiral from Diagnostica Stago, Asnières, France. One unit of vWF activity was defined as the amount present in 1 mL of normal pig pooled plasma.

vWF Multimeric Structure

vWF multimer analysis was carried out by horizontal SDS-agarose gel electrophoresis using 1% agarose gels. A rabbit anti-human-vWF antiserum (No. A082, DAKO), which strongly cross-reacts with porcine vWF, was used as the primary antibody. Alkaline phosphatase–conjugated affinity-purified goat anti-rabbit IgG (Axell Accurate Chemical and Scientific Corp) was used as the secondary antibody. Staining was carried out with a nitro blue tetrazolium chloride/bromochlor indolyl phosphate substrate system.

SLA Determination

SLA class 1 was determined by serology. Compatibility in class 2 was ascertained by mixed lymphocyte culture.46,48

Bleeding Time

Saline ear bleeding time was measured according to Mertz.49 Briefly, the marginal edge of the ear was quickly transfixed (standardized
incision of 3 mm length through the entire thickness of the ear) with a surgical blade (No. 11). The ear was immediately placed into a beaker containing 500 mL of citrated saline solution (20 mmol/L sodium citrate and 150 mmol/L NaCl) at 37°C. Time was recorded at the end of bleeding as 30 minutes if bleeding had not spontaneously stopped by this time.

**Histological Study**

Histological examination was performed on 3-μm paraffin sections stained by hematoxylin-eosin-saffron and Masson’s trichrome. Lung rejection was evaluated according to 2 histological classifications.50,51

**Immunohistochemistry of vWF**

Immunohistochemistry was performed on 3-μm tissue sections by the indirect alkaline phosphatase anti–alkaline phosphatase (APAAP) method52 with fast-red TR salt (DAKO APAAP kit, system 40, K670) used for development or by the avidin-biotin peroxidase complex method with 3,3′-diaminobenzidine used for development. A polyclonal rabbit anti-human vWF (No. A082, DAKO) cross-reacting with porcine vWF (according to DAKO specifications and personal data31) and diluted to 1/400 was used. Omission of primary antibody was used as a negative control. For homogeneity of labeling, immunohistochemistry was performed with an automatic immunostainer (Histostainer, Leica).

**Quantification**

Intensity of labeling was semiquantitatively evaluated according to a 0 to 4 graded scale: 0 indicates no staining; 1, very weak staining, only perceptible at ×40 magnification; 2, weak staining, perceptible at ×25 magnification; 3, moderate staining, perceptible at ×10 magnification; and 4, strong staining, perceptible at ×2.5 magnification. Heterogeneity of the labeling was designated “v” when labeling varied from vessel to vessel and “Sc” when scattered positive cells were disseminated among negative blood vessels.

**Results**

**Clinical Evaluation**

Pig 1 died from intrathoracic hematoma 7 days after surgery, and pig 10 died from postoperative acute pulmonary edema (see Table for characteristics of pigs used for study). For other animals, including the pig that had undergone left pneumonectomy, the postoperative course was uneventful, and they were killed for study between 7 and 209 days after surgery. At that time, these animals did not appear to have any pathological clinical symptoms.

**Technetium perfusion scintigraphy** was performed in transplanted vWF pigs 2 and 5 at day 23 and day 72, respectively, and in the normal transplanted pig 8 at day 64, which confirmed normal perfusion of the left transplanted lung.

**Autopsy Findings**

In pigs 3, 5, 6, and 7, portions of the left lung were reduced to necrotic material surrounded by a thick fibrous wall. Transplantation failure was due to stenosis of the left bronchial anastomosis and/or of the left pulmonary artery and/or pulmonary veins.

In other transplanted pigs (Nos. 2, 4, 8, 9, 11, 12, and 13), sutures of the left bronchus, pulmonary artery, and pulmonary veins were unremarkable, and the lung parenchyma was macroscopically normal or with little foci of edema but without any foci of necrosis. These animals successfully transplanted were used for histological and immunologic studies.

**Histological Examination**

In control lungs (explanted recipient left lungs and donor right lungs), minor pathological changes of lung parenchyma were observed. Thus, some foci of edematous alveolitis were seen; these were probably due to surgery. Moreover, bronchus-associated lymphoid tissue was particularly well developed.53 It was organized as peribronchiolar groups of small lymphocytes, sometimes forming
lymphoid nodules, or as small heterogeneous lymphocyte aggregates in alveolar walls.

After Transplantation
In the recipient native right lung, foci of edematous alveolitis with macrophages were more numerous and bronchus-associated lymphoid tissue was as much developed as in control lungs.

SLA-Compatible Transplanted Left Lungs
Lungs from pigs 2, 4, and 8, killed at days 71, 48, and 64, respectively, exhibited the same histological features as control lungs.

In pigs 9, 11, and 12, killed at days 22, 18, and 38, respectively, foci of acute alveolitis and perivascular lymphocyte infiltrates were found and were sufficient to diagnose minor acute lung rejection grade 1 according to Clelland et al. or grade A1a-A1ac according to Youssem et al. 51

SLA-Incompatible Transplanted Left Lung
Pig 13, killed at day 6, exhibited histological signs of severe acute lung rejection grade 3 (Clelland et al.) or grade A3ac (Youssem et al.) with perivascular and peribroncholar infiltrates of small lymphocytes admixed with some immunoblasts, extending in the alveolar septa. This was associated with extensive foci of alveolitis.

Plasma vWF

Normal Pigs After Left Pneumonectomy
Before pneumonectomy, plasma vWF level was 100 U/dL. After lung removal, plasma vWF decreased in the first postoperative days to reach a plateau of 86 U/dL (−14%).

Normal Pigs Transplanted With SLA-Compatible Normal Lung (Pigs 8 and 12)
In pig 8, without sign of rejection, plasma vWF increased during the 8 days after transplantation (+61%) and then slowly recovered to the normal initial value (Figure 1). In pig 12, with minor histological signs of rejection, plasma vWF increased during the first postoperative day (+39%) and then decreased to a lower value (−24%) (Figure 1).

Homozygous vWD Pigs Transplanted With SLA-Compatible Normal Lung (Pigs 2, 4, 9, and 11)
Plasma vWF level increased in the postoperative hours and appeared to have a partially corrected vWF defect, which was presumably due to the infusion of vWF concentrate to reduce perioperative blood loss. Then, plasma vWF level decreased to reach a low but significantly detectable plateau of 6.4 and 6.2 U/dL in cases without minor histological sign of rejection (pigs 2 and 4, respectively) and 4.8 and 2 U/dL in cases with minor rejection (pigs 9 and 11, respectively) (Figure 2).

Normal Pig Transplanted With SLA-Incompatible Normal Lung (Pig 13)
In pig 13, plasma vWF level increased in the first postoperative hours from 88 to 108 U/dL and then recovered normal initial value (82 U/dL).

vWF Multimers
vWF multimeric analysis was performed in normal donors, in vWD recipients before transplantation, and in transplanted vWD pigs 1 and 2 months after lung transplantation (Figure 3).

In normal donors, full ranges of vWF multimers with normal distribution were found. In vWD recipients, no detectable vWF was found. In the postoperative period, all types of vWF multimers were detected with a normal pattern. In successful transplantations (pigs 2 and 9), all ranges of vWF multimers with a normal distribution were detected 1 and 2 months after transplantation. The failed transplantation (pig 6) exhibited only traces of vWF, with a deficit in high molecular weight multimers.

Bleeding Time
In normal pigs transplanted with SLA-compatible or -incompatible normal lungs and in the normal pig after pneumonectomy, bleeding time was normal (<5 minutes) and remained normal (<5 minutes).
Figure 3. Plasma vWF multimeric analysis of transplanted vWD pigs (pigs 2 and 6; see Table). Lanes are as follows: lanes 1 to 5, pig 6, failed transplantation; lanes 6 to 10, pig 2, successful transplantation; lanes 1 and 6, normal donors before transplantation, with normal multimeric pattern of plasma vWF; lanes 2 and 7, vWD recipient before transplantation, with no detectable plasma vWF multimers; and lanes 3 and 8, transplanted vWD pig in postoperative period (all types of vWF multimers are detected with normal multimeric pattern that is due to infusion of vWF concentrate for antihemorrhagic conditioning). Failed transplantation is shown at 1 month (lane 4) and 2 months (lane 5) after transplantation; only traces of vWF are found with a deficit of high molecular plasma vWF multimers. Successful transplantation is shown at 1 month (lane 9) and 2 months (lane 10) after transplantation: all ranges of plasma vWF multimers with a normal pattern are found.

In homozygous vWD pigs transplanted with SLA-compatible normal lung, bleeding time lasted >30 minutes and was not changed by the transplantation of normal lung containing vWF.

Immunohistochemistry of vWF

Normal Pig Lungs (Control Lungs)

All ECs from peribronchial and mucosal capillaries and veins exhibited strong granular intracytoplasmic labeling for vWF. Except for ECs from pulmonary arteries, which were strongly stained, ECs from arteries displayed heterogeneous staining for vWF, characterized by moderate granular cytoplasmic labeling of equal intensity of sparsely stained ECs disseminated among negative ones. All pulmonary artery ECs displayed strong labeling. Pulmonary capillary ECs were moderately to weakly stained, with variation from one EC to another and from one blood vessel to another. Alveolar capillary ECs, which represent the major EC mass in the lung, stained weakly or not at all.

vWD Pig Lung

No significant labeling of vWF could be detected.

Normal SLA-Compatible Transplanted Lungs (Pigs 2, 4, 8, 9, 11, and 12)

In pigs 2, 4, 8, 9, 11, and 12, vWF was detected in the same location as in control lungs, but the intensity of labeling was weaker and more heterogeneous. As in control lungs, ECs of peribronchial and mucosal capillaries expressed the greatest amount of vWF (Figure 4, panels a and b).

Normal SLA Incompatible Transplanted Lung (Pig 13)

In pig 13, vWF was detected in the same locations as in control lungs. The intensity of EC labeling was stronger and more diffuse than in control lungs (Figure 4, panels c and d).

Discussion

Because the circulating plasma vWF mainly originates from ECs and because pig lung contains numerous ECs with striking heterogeneous vWF expression, lung transplantation between normal and vWD pigs is an excellent approach to study vWF synthesis and release from transplanted ECs in vivo.

Initially, the effect of left lung removal on plasma level of vWF was evaluated. Left pneumonectomy induced a prolonged decrease of plasma vWF (−14%). This provided 2 types of information: vWF production by remnant ECs cannot compensate for the deficit, and basal plasma vWF probably depends on the mass of secretory ECs.

In successful lung transplantations, including SLA-incompatible transplantation, ECs are morphologically intact, and their function, reflected by vWF production and normal multimeric plasma vWF pattern, is preserved, indicating that ECs are viable after transplantation.

One of the peculiarities of this model is the use of SLA-compatible pigs, which allows deletion of immunosuppressive therapy, which is toxic for ECs and may affect vWF synthesis. In these conditions, even in the absence of signs of clinical rejection, features of minor histological rejection were observed in 3 of the 6 successful lung transplantations. This indicates that SLA-compatible pigs may develop minor rejection and that systems other than SLA (minor early antigens) are involved in the occurrence of minimal histological rejection.

In the present study, the EC vWF production was modulated differently according to the relation of donor to recipient. In SLA-compatible normal lungs transplanted to normal pigs or to vWD pigs, the EC vWF storage pool—reflected by a decrease of vWF EC content—was excreted and/or deviated to the constitutive secretory pathway. Moreover, when a normal lung was transplanted to a vWD pig, there was no recruitment of ECs that initially did not secrete vWF, in order to palliate vWF deficiency.

In SLA-compatible transplantations without histological signs of rejection, EC vWF excretion was able to maintain normal plasma vWF levels in the case of transplantation between normal pigs (No. 8) and to partially correct plasma vWF level in transplanted vWD pigs (Nos. 2 and 4), in the amount expected from the transplanted tissue only.

In lungs with minor histological signs of rejection, an alteration of vWF synthesis with probable constitutive pathway alteration was suggested, in view of the fact that plasma vWF levels were slightly decreased in the case of transplantation between normal pigs (No. 12) and partial correction of plasma vWF level was less apparent (Nos. 9 and 11) than in lungs exhibiting no rejection (Nos. 2 and 4).

Thus, this model allows the investigation of the vWF secretory functions of ECs when they are transplanted into another animal, notably devoid of vWF.

Because of their location, ECs play an important role in graft rejection. Just after an organ transplant, antigenic determinants associated with major histocompatibility complex of the donor stimulate T lymphocytes of the recipient to induce immunologic reactions that can lead to graft rejection. During graft rejection, ECs of the transplanted organ may act as an active participant or as an immune target. As a target, they are the first cells exposed to humoral factors and are in contact with circulating leukocytes of the immunocompetent recipient. As an active participant of graft immune reaction, ECs can interact with recipient leukocytes, initiating recruitment of recipient immunocompetent cells. Several steps are
necessary for this recruitment: (1) accumulation of effective cells in the graft microcirculation, (2) adhesion of effective cells to ECs, and (3) migration of immune cells into the surrounding connective tissue. In all these steps, an important role seems to be played by adhesion molecules and cytokines.2–4

In incompatible lung transplantation between normal pigs, histological signs of severe rejection were found, as expected. ECs were morphologically normal, and their activation was reflected by an increased vWF EC content, as has been described in ECs of human endomyocardial biopsies.4 This feature is observed only in ECs that initially produced vWF; there is no recruitment of other ECs even if they are activated. This was not associated with a rise of plasma vWF and argues that the release of vWF from ECs in the plasma is regulated to stay in the normal range and that the constitutive pathway is probably deviated to the storage pathway.

vWF, and notably plasma vWF, has been demonstrated to be involved in the arrest of bleeding in pigs.57 Absence of bleeding time correction in homozygous vWD-transplanted pigs may be due to insufficient increase of plasma vWF and, subsequently, of coagulation factor VIII and/or lack of plasma vWF incorporation in vWD pig platelets, as previously reported in a bone marrow graft model.58

Pigs have been used for the evaluation of various surgical techniques59 and for the study of mechanisms of rejection, such as obliterans bronchiolitis.60 The present study demonstrates that vWD pigs can be used in major surgical protocols, such as lung transplantation, if they are blood- and vWF-substituted in the operative period.

In this transplantation protocol, circulation of bronchial arteries was not restored, although peribronchial, mucosal, and submucosal capillaries were lined by viable and morphologically unaltered ECs. The fact that ECs from bronchial-dependent circulation keep their vWF expression and react as ECs from pulmonary-dependent circulation argues in favor of preservation of the function of this circulation. This may be related to the presence of pulmonary-systemic collaterals in successful transplantations. Lack of or poor development of this circulation may explain the occurrence of bronchial stenoses and subsequent transplantation failure. Restoration of bronchial-dependent circulation, substitutive transplantation of omentum around the bronchial suture area, or the use of metallic stents may overcome this problem, as described in humans.61–63

Finally, the present study demonstrates that pig lung transplantation is a good model for studying the regulation of EC synthesis in vivo because, physiologically, pig lung ECs
exhibit a striking heterogeneity of vWF expression between pulmonary and bronchial circulations.

In both compatible and incompatible successful transplantations, ECs maintain viability and function reflected by their ability to produce vWF, even if they are transplanted into an animal devoid of vWF. The vWF constitutive and storage pathways are modulated differently according to transplantation compatibility and to the severity of histological signs of rejection. This model provides a good approach to understand the regulation of vWF by ECs after transplantation, but it cannot be directly extrapolated to the EC regulation of vWF in human organ transplantation because, besides species differences, humans receive immunosuppressive treatment, which may be toxic to ECs.

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