



## Differential aspects of the glycoprotein Ib-von Willebrand factor axis in human and pig species

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### ABSTRACT

**Background and Objectives.** The role of glycoprotein Ib (GPIb) in platelet adhesion to subendothelium is well established in human species. However, the interaction of GPIb and von Willebrand factor (VWF) in a widely used experimental model in thrombosis research, that of the pig, has not been clearly elucidated. We investigated the differences between human and pig species in the GPIb/VWF axis in several ways.

**Design and Methods.** Standard aggregometry and perfusion studies with circulating blood were applied to isolated platelets or to blood reconstituted with isolated platelets, VWF and red blood cells from the different species. Platelet aggregation to VWF in the presence of either ristocetin or botrocetin was tested.

**Results.** Human VWF and ristocetin did not agglutinate pig platelets. However, botrocetin was capable of agglutinating pig platelets. In perfusion studies (800 s<sup>-1</sup>, 10 min), washed platelets from both species were suspended in albumin solutions containing human VWF (hVWF) or porcine VWF (pVWF) and red blood cells from the corresponding species. Reconstituted blood with high concentrations of pVWF ( $\geq 0.25$  U/mL) caused severe thrombocytopenia during the perfusion procedure when added to human platelets. Nevertheless, lower concentrations ( $\leq 0.1$  U/mL) promoted the formation of large aggregates. Under our experimental conditions, hVWF poorly supported pig platelet adhesion.

**Interpretation and Conclusions.** In conclusion, pVWF may support human platelet adhesion and even promote aggregation, while hVWF can only partially facilitate pig platelet adhesion. Minimal concentrations of pVWF could facilitate the interaction of human platelets with subendothelium, increasing their adhesive and aggregating capabilities. Understanding the molecular differences of the GPIb-VWF axis in different species may prove useful for developing therapeutic strategies aimed at preventing excessive platelet deposition on damaged vascular surfaces.

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Key words: experimental model, platelets, von Willebrand factor, glycoprotein Ib, subendothelium

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Cardiovascular disease accounts for about 35% of total mortality in developed countries. Coronary artery disease and its complications are the main contributors to this pathologic condition, and platelets are known to play an important role at different stages of its development.<sup>1</sup> Mechanisms leading to the establishment of platelet aggregates on damaged vessels causing arterial occlusion and sudden ischemia are well characterized in human species. The interaction of platelet glycoprotein Ib (GPIb) with von Willebrand Factor (VWF) exposed on vessel subendothelium is the crucial event for platelet attachment onto damaged vessel surfaces.<sup>2</sup>

The pig is frequently used as an experimental model in the investigation of the underlying mechanisms in thrombosis, as well as in the search for new therapeutic agents.<sup>3,4</sup> However, it is known that porcine models present singularities in mechanisms related to platelet adhesion processes when studies are performed in citrated blood.<sup>5</sup>

Ristocetin is an antibiotic that binds to the 509-695 amino acid sequence of human VWF (hVWF), facilitating its binding to GPIb and further agglutination of platelets.<sup>6</sup> Ristocetin does not induce conformational changes in porcine VWF (pVWF) that could facilitate its interaction with GPIb. Interestingly, botrocetin can modify both porcine and hVWF, thus facilitating its binding to porcine GPIb.<sup>7,8</sup>

The distinct behaviors of human and pig species in responses to ristocetin and botrocetin are likely related to the differential molecular structures of VWF and/or GPIb. A possible explanation for these differences could be that pVWF has certain molecular alterations that make it less reactive to porcine GPIb. However, the latter suggestion of a reduced reactivity of pVWF seems in contradiction with its well-known ability to cause spontaneous agglutination of human platelets, even in the absence of ristocetin.<sup>8,9</sup> The present study was designed to characterize the role of the GPIb/VWF axis in pig species. For this purpose we incubated isolated platelets from human or pig species with homologous or heterologous VWF. Agglutinating responses to botrocetin and ristocetin were tested in the aggregometer. The reactivity of platelets in the incubates towards subendothelial elements was analyzed under flow conditions in perfusion experiments at intermediate shear rates and performed in all cases in the presence of red blood cells.

## Design and Methods

### Animals

Six normal male and female pigs, Landrace and Large White, 3-4 months old with an average weight of  $30.7 \pm 3.7$  Kg (range 25-32 kg) purchased from local farmers and fed a normal chow diet, were used in our experiments.

The investigation complied with the *Principles of animal care* published by the US National Institute of Health (NIH #86-23, revised 1985) and with our Institution's guidelines.

### Study design

Pigs were sedated with intramuscular azaperone 2 mg/kg. An ear vein was cannulated and thiopental 30 mg/kg was administered. The pigs were intubated and mechanically ventilated with oxygen by a pressure respirator (Bird mark 8, Palm Spring, CA, USA); anesthesia was maintained with iv thiopental. An arterial sheath was inserted into the right femoral artery to obtain blood samples.

Human blood was obtained from healthy voluntary donors who had not taken aspirin in at least the preceding two weeks.

### Blood sampling and study strategies

Porcine blood samples were obtained from cannulation of the pig femoral artery. Human samples were extracted from the antecubital vein. Blood samples were rapidly mixed with citrate-phosphate-dextrose (CPD).

Platelets, plasma and red blood cells from the total blood volume were isolated following standard methodology.<sup>10</sup> Samples were divided into aliquots in order to perform aggregation tests and to analyze cross-responses to hVWF and pVWF.<sup>11</sup>

### Platelet isolation

Pig and human platelets were isolated following standard methodology.<sup>10</sup> Blood samples were centrifuged (200 g, 10 min) in order to obtain platelet-rich plasma (PRP). The PRP was suspended, following a 1:1 proportion (v:v), into citrate-citric-dextrose (CCD) buffer (sodium citrate 93 mM, citric acid 7 mM, dextrose 140 mM) (pH=6.5) in the presence of platelet activation inhibitors, adenosine (5 mM) and theophylline (3 mM). After adding CCD, the platelet suspension was centrifuged at 1,800 g for 20 minutes. The supernatant was eliminated and platelets were isolated from co-sedimented red cells. This process was repeated twice in order to isolate platelets completely. After the last centrifugation, the platelet pellet was suspended into Hank's balanced salt solution (pH=7.2). The platelet concentration was adjusted to  $1.2 \times 10^6$  platelets/ $\mu$ L for perfusion studies and  $4 \times 10^5$  platelets/ $\mu$ L for aggregation studies.

Human and pig platelet-poor plasma (PPP) as well as red blood cells were also obtained following standard methodology.<sup>10</sup> After obtaining the PRP, the rest of the sample was centrifuged at 1,200 g (20 min, 4°C) and PPP was separated. Red blood cells were obtained by washing the red pellet with red cell washing buffer (0.9% NaCl, 0.1% glucose).

### Aggregation studies

The ability of platelets to aggregate was tested by conventional turbidimetric procedures.<sup>12</sup> Aliquots of pig and human platelets were suspended in albumin solution containing pig (pVWF) or human (hVWF) VWF. Agonists tested were ristocetin (1 mg/mL) and a South-American snake venom (*Botrox Jararaca*) with procoagulant properties called botrocetin (10  $\mu$ g/mL).

### Perfusion studies

Blood samples were reconstituted by suspending isolated platelets and red blood cells in 1) albumin solution (PPL), 2) albumin solution containing pVWF or hVWF.

Perfusions were carried out at 37°C in perfusion chambers as developed by Baumgartner<sup>13</sup> with an effective annular width of 2.2 mm and a rod length of 7.2 cm. Flow was obtained by pumping blood through a peristaltic pump. Blood was perfused at 140 mL/min (wall shear rate of 800 s<sup>-1</sup>).  $\alpha$  chymotrypsin-denuded rabbit aortas were used in all the experiments.<sup>14</sup> Aortic segments were mounted on plastic rods and rinsed with phosphate-buffered saline (PBS) for 10 min. After 10 min of perfusion with the blood samples, segments were rinsed with the same phosphate buffer and fixed with 3% glutaraldehyde.

After 1 h, segments were separated from the rod, washed with PBS (BioMérieux, France), dehydrated through a grade series of ethanol, embedded in JB-4 embedding material (Polysciences, Warrington, PA, USA), thin sectioned for light microscopy and stained with methylene blue.

### Morphometric evaluation

Platelet interactions with subendothelium were morphometrically evaluated using a computerized image analysis system.<sup>15</sup> The morphometric parameters were determined according to the criteria previously established by Baumgartner.<sup>14</sup> Briefly, platelets or groups of platelets were classified as: 1) contact (C), platelets that are attached to but not spread on the subendothelium; 2) adhesion (A), platelets that spread on the subendothelial surface, including small aggregates of less than 5  $\mu$ m in height; 3) thrombi (T), platelet aggregates of more than 5  $\mu$ m in height. All these basic parameters were expressed as a percentage of the total examined surface. Another parameter related to those previously defined, was the total covered surface (CS), which was determined by adding C+A+T.

### Statistical analysis

Student's t-test was used for statistical comparisons. The results are expressed as mean $\pm$ SD. A *p*-value of < 0.05 was considered statistically significant.

## Results

### Studies in platelet suspensions

Washed human and pig platelets suspended in albumin solution containing human or pVWF, were activated in the presence of ristocetin (1 mg/mL) and botrocetin (10  $\mu$ g/mL). Human platelets agglutinated in the presence of hVWF and ristocetin. As expect-

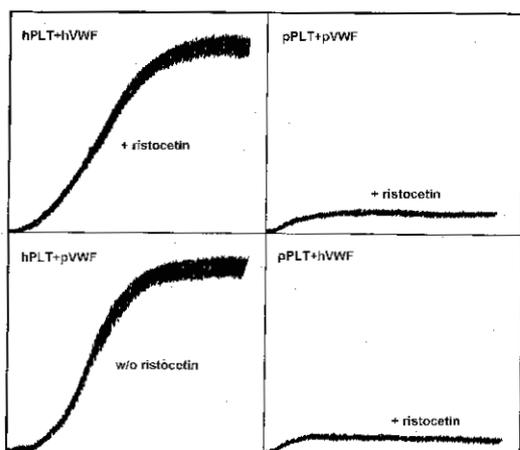


Figure 1. Comparative analysis of maximal aggregation patterns obtained using human (hPLT) or pig platelet (pPLT) samples in the presence of human (hVWF) or pig (pVWF) von Willebrand factor. Ristocetin (1 mg/mL) was used as the aggregating agent. Pig von Willebrand factor was able to agglutinate human platelets in the absence of ristocetin. However, pig platelets did not respond to ristocetin-activated human von Willebrand factor.

ed, ristocetin was unable to induce any agglutination in pig platelets when pVWF was present (Figure 1). Response was also absent when pig platelets were put together with ristocetin-activated hVWF (Figure 1). These last data would agree with a lack of reactivity of pig GPIb even to ristocetin-activated hVWF.

However, human platelets spontaneously agglutinated in the presence of pVWF, without the addition of ristocetin (Figure 1), thus confirming an increased reactivity of human GPIb to pVWF or an increased affinity of pVWF for human GPIb.

Pig platelets only agglutinated when VWF was activated with botrocetin, in the presence of either human or pVWF.

### Studies under flow conditions

The interaction of human platelets suspended in albumin solution lacking VWF was severely impaired (Figure 2). Under similar experimental conditions, pig platelets retained levels of adhesion that were significantly superior ( $p=0.003$ ) to those observed with human platelets (Figure 3). A marked improvement in human platelet adhesion was observed in those perfusion experiments in which 0.6-0.8 U/mL of hVWF were added to the albumin solution (Figure 2). In contrast with the previous observation, hVWF caused only a very mild improvement in the adhesion of pig platelets (Figure 3).

The presence of pVWF at concentrations  $\geq 0.25$  U/mL in the albumin solution containing human platelets spontaneously caused platelet agglutination, leading to thrombocytopenia. Under these experimental conditions, perfusion studies showed very decreased levels of interaction (Figure 2). How-

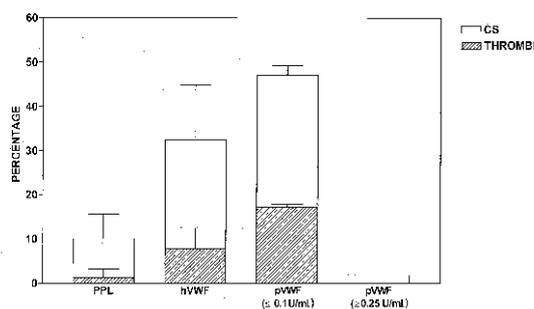


Figure 2. Bar diagram showing percentages of the sub-endothelial covered surface (empty bars) and the percentages of thrombi formation (dashed bars) by human platelets under different experimental perfusion conditions ( $n=6$ ) (PPL: albumin solution).

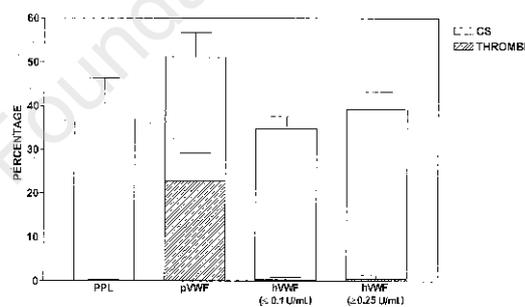


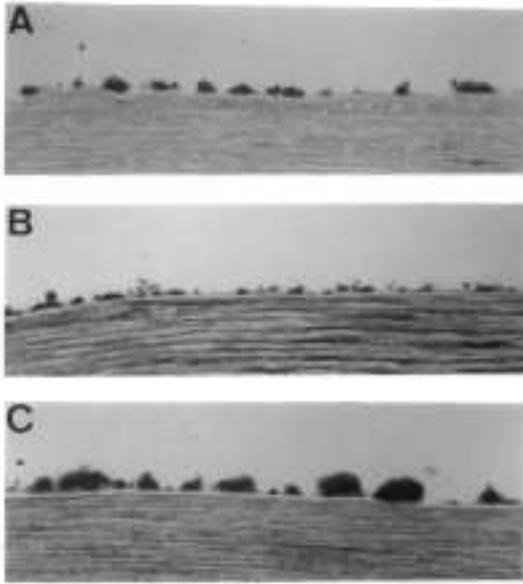
Figure 3. Bar diagram showing percentages of the sub-endothelial covered surface (empty bars) and the percentages of thrombi formation (dashed bars) by pig platelets under different experimental conditions ( $n=6$ ) (PPL: albumin solution).

ever, when lower concentrations of pVWF ( $\leq 0.1$  U/mL) were used, an improvement in human platelet interaction with the subendothelium was observed (Figures 2 and 4).

### Discussion

Pig species are widely used as an experimental model in the investigation of pathophysiological mechanisms of athero-thrombosis.<sup>3,16</sup> The evident physiologic similarities at vascular level between human and pig species have greatly contributed to the development of our knowledge about mechanisms underlying cardiovascular diseases and possible therapeutic strategies.<sup>17</sup> The present work has identified distinct responses of human and pig platelets to VWF that suggest peculiarities in the function of the GPIb-VWF axis in both species.

The establishment of adhesive contacts between



**Figure 4.** Light micrographs (400x) obtained from cross-sections of everted segments of rabbit aortas perfused with (A) reconstituted human blood, (B) reconstituted pig blood, (C) reconstituted human blood in the presence of pVWF (0.1 U/mL). There was an increase in human platelet-subendothelium interaction when low concentrations of pVWF were present.

platelets and subendothelium is an essential process in the development of correct hemostasis, though it also plays an important role in the initiation of thrombotic complications. Primary adhesion of platelets to subendothelium is mediated by the interaction of vessel-wall VWF with platelet GPIb.<sup>18,19</sup> In human species, there are about 20-25,000 copies of GPIb. This glycoprotein forms a complex with GPV and GPIIX,<sup>20,21</sup> which guarantee the adhesive function of platelets.

The A1 domain of hVWF – more specifically the 509-695 amino acid sequence – is thought to be responsible for its interaction with GPIb.<sup>6</sup> It is well established that proteolytic fragments of VWF that preserve A1 domain bind spontaneously to human GPIb. Ristocetin induces an alteration of the 474-488 and the 694-708 peptide sequences that are close to A1 domain. This alteration leads to a conformational change in the molecule of VWF, increasing its affinity for human GPIb. It has been demonstrated that peptides containing an Asp514-Glu542 sequence inhibit the binding of VWF to human GPIb.

In our studies, pig platelets did not respond to ristocetin, as previously described in literature, but were able to be activated by botrocetin. Botrocetin acts on the 539-553 region of the A1 domain, inducing a conformational change in VWF and allowing its binding to pig platelet GPIb.<sup>22</sup> It is interesting to note that pVWF that is not activated by ristocetin, needing botrocetin to change its conformation in order to

interact with pig GPIb, spontaneously binds to human GPIb thus agglutinating human platelets in the absence of ristocetin or botrocetin. The most suitable explanation is that pVWF has structural differences that make it less reactive to pig GPIb, but highly reactive to human GPIb. The fact that hVWF did not bind to pig GPIb, even after activation with ristocetin, confirms that pig GPIb possesses less affinity to hVWF. All these data suggest that the GPIb-VWF interaction might be less critical for the adhesive platelet events in pig species. Results of our perfusion studies in which pig platelets suspended in an albumin solution, in the absence of VWF, still covered 36% of the vessel surface suggest that other receptors could also contribute to the initial attachment of pig platelets onto damaged vascular surfaces.

Under the experimental conditions used in our perfusion experiments, hVWF seemed far from being the ideal adhesive protein for pig platelets. The presence of hVWF in the perfusates only caused a very mild improvement in the adhesion of pig platelets in our experiments. It should be mentioned that our studies were performed at moderately high shear rates (800 s<sup>-1</sup>) and that VWF seems to play a more important role in platelet adhesive events when shear rates are greater.<sup>23</sup> In fact, previous studies have demonstrated that infusion of hVWF improves hemostasis in pigs severely deficient in VWF under high shear rate conditions.<sup>24</sup>

Data obtained in the present study seem to indicate that pVWF could share some similarities with that of human von Willebrand's disease 2B or asialo VWF. Type 2B von Willebrand's disease refers to qualitative variants in the molecule of VWF that make it more reactive for platelet GPIb.<sup>25</sup> This abnormal VWF spontaneously binds to platelets, which can lead to the loss of high molecular weight multimers of plasma VWF and episodes of thrombocytopenia. It has also been demonstrated that the chemical desialylation of hVWF results in molecules with the ability to bind spontaneously to human GPIb.<sup>26</sup> In our studies performed under flow conditions, the presence of high concentrations of pVWF led to spontaneous agglutination of human platelets during the perfusion procedure with diminished vascular coverage. In contrast to this finding, lower concentrations of pVWF ( $\leq 0.1$  U/mL) were able to support not only human platelet adhesion but also formation of platelet aggregates. Studies by Gralnick *et al.* using asialo-VWF as a source of VWF in perfusates found decreased platelet interaction in perfusion studies.<sup>27</sup> Contemporary studies by Bastida *et al.* found exactly opposite results.<sup>28</sup> Our experience with pVWF may help to explain the controversial results of the two groups based on the different concentrations of asialo-VWF used in the experiments. Moreover, our findings suggest that a low concentration of VWF together with molecular alterations such as those naturally occurring in pig species, could be prothrombotic in humans. In fact, porcine VWF may contaminate porcine FVIII concentrates that are currently used in the control of hemorrhagic episodes in hemophilic patients developing inhibitors.

The studies suggest the existence of certain singularities in some functional aspects of the platelets of

the pig model that could affect adhesion phenomena mediated through GPIIb-VWF interactions.<sup>5,26,29,30</sup> It is very likely that during the evolution process, pig species would have developed their specific hemostatic mechanisms that may differ from those of other mammals.

In relation to those glycoproteins regulating adhesion, our study directly implies that pig GPIIb has structural differences with respect to human GPIIb, making it less reactive to VWF. This fact has no dramatic impact on the hemostasis of this species, because of the existence of other compensatory mechanisms.

### Contributions and Acknowledgments

*MJZ was the principal investigator; she carried out the experimental part of the study and the statistical analysis. GE was responsible for handling and interpreting data and direct supervision. MJZ and GE wrote the paper and, with MH, were responsible for the design of the study. MH contributed to the execution of the study by providing pig blood samples. We thank AO and RC for their contribution to the conception of the discussion of the present manuscript. AO was responsible for the funding and facilities, making the accomplishment of this work possible.*

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### Disclosures

*Conflict of interest: none.*

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### Manuscript processing

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### Potential implications for clinical practice

- ◆ Our data support the concept that minimal amounts of VWF with structural variations might promote thrombosis.
- ◆ Investigation of the regulation of the GPIIb/VWF axis in different species, could be of interest for the development of therapeutic strategies aimed at controlling excessive platelet reactivity.

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