The infection of microvascular endothelial cells with ExoU-producing *Pseudomonas aeruginosa* triggers the release of von Willebrand factor and platelet adhesion

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An increased plasma concentration of von Willebrand factor (vWF) is detected in individuals with many infectious diseases and is accepted as a marker of endothelium activation and prothrombotic condition. To determine whether ExoU, a Pseudomonas aeruginosa *cytotoxin with proinflammatory activity, enhances the release of vWF, microvascular endothelial cells were infected with the ExoU-producing PA103* P. aeruginosa *strain or an* exoU*-deficient mutant. Significantly increased vWF concentrations were detected in conditioned medium and subendothelial extracellular matrix from cultures infected with the wild-type bacteria, as determined by enzyme-linked immunoassays. PA103-infected cells also released higher concentrations of procoagulant microparticles containing increased amounts of membrane-associated vWF, as determined by flow cytometric analyses of cell culture supernatants. Both flow cytometry and confocal microscopy showed that increased amounts of vWF were associated with cytoplasmic membranes from cells infected with the ExoU-producing bacteria. PA103-infected cultures exposed to platelet suspensions exhibited increased percentages of cells with platelet adhesion. Because no modulation of the vWF mRNA levels was detected by reverse transcription-polymerase chain reaction assays in PA103-infected cells, ExoU is likely to have induced the release of vWF from cytoplasmic stores rather than vWF gene transcription. Such release is likely to modify the thromboresistance of microvascular endothelial cells.*

Key words: *Pseudomonas aeruginosa* - von Willebrand factor - prothrombotic activity - microparticles

Pseudomonas aeruginosa is a major agent of sepsis, a clinical syndrome characterised by the infection-triggered activation of the inflammatory and coagulation systems and by endothelial dysfunction (Esmon 2005).

The endothelium usually functions as an antithrombotic surface and is critical for maintaining normal vascular homeostasis (Schouten et al. 2008). However, under conditions of sepsis, endothelial cells are activated to secrete and express surface procoagulant molecules such as tissue factor (TF) and von Willebrand factor (vWF) (Vallet & Wiel 2001). Activated endothelial cells also release increased amounts of procoagulant microparticles, which are small membrane-derived vesicles shed by cells following activation or during apoptosis under a variety of pathophysiological circumstances (Martinez et al. 2005). Microparticle membranes exhibit an antigen repertoire that is representative of the cell membranes from which they are derived, except for the loss of phospholipid asymmetry. Consequently, microparticle membranes contain anionic phosphatidylserine in their exoplasmic leaflet, enabling the assembly of clotting enzyme complexes and the generation of thrombin. Hardly detectable in the peripheral blood of healthy individuals, elevated levels of circulating procoagulant microparticles are often associated with thrombotic potential. The expression of TF (del Conde et al. 2005) and vWF (Jimenez et al. 2003) on endothelial microparticle membranes further contributes to their potential role in hemostasis dysfunction.

VWF is a large multimeric glycoprotein that is synthesised in endothelial cells and megakaryocytes and plays a pivotal role in hemostatic plug formation and thrombosis (Ruggeri 2007). The main functions of vWF are to mediate the adhesion of platelets to sites of vascular injury and to act as a bridge molecule for platelet aggregation, promoting the interaction of specific binding sites with the platelet surface receptors GP Iba and aIIbb-3 integrin (Ruggeri 2007, Varga-Szabo et al. 2008). High-molecular-weight vWF multimers, the most important factors for physiological platelet function, are released from endothelial cells towards both the lumen and the subendothelial matrix (Ruggeri 2003, 2007). Increased concentrations of circulating vWF are detected in individuals with many pathologic conditions, including acute infectious diseases (Pottinger et al. 1989,, Kayal et al. 1998), and are generally accepted as a marker of endothelium activation/dysfunction (Vischer et al. 2000).

One of the most important *P. aeruginosa* virulence factors is ExoU, a cytotoxin with phospholipase A_2 activity (Sato et al. 2003, Engel & Balachandran 2009). Pre-

Financial support: CNPq, FAPERJ
CF was supported by a scholarship from CNPq.
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Received 8 November 2011
Accepted 9 April 2012

vious studies from our group have highlighted the ability of ExoU to elicit a potent inflammatory response in microvascular endothelial cells (Saliba et al. 2005) and to enhance the release of soluble ICAM-1 from *P. aeruginosa*-infected endothelial cells (Lins et al. 2010), demonstrating the toxin's ability to induce endothelial activation/dysfunction (Gando et al. 2005). In the present study, we investigated whether ExoU would promote the release of vWF from endothelial cells, thereby contributing to enhanced platelet adhesion/aggregation and potentially to the generation of prothrombotic conditions.

SUBJECTS, MATERIALS AND METHODS

Bacterial strains and culture conditions - *P. aeruginosa* PA103 and the ExoU-deficient PA103Δ*exoU* mutant (Saliba et al. 2005) were used throughout this study. Bacteria were grown in Luria-Bertani broth at 37ºC for 16-18 h, harvested by centrifugation and resuspended in MCDB 131 cell culture medium (Sigma-Aldrich) supplemented with 10% foetal calf serum, 10 ng mL^{-1} EGF (Sigma-Aldrich), 1 μg mL^{-1} hydrocortisone (Sigma-Aldrich) and glutamine (complete culture medium).

Cell culture and infection - Cells from the human microvascular endothelial cell line-1 (HMEC-1) (Ades et al. 1992) were cultured in complete MCDB-131 culture medium for 48 h. Bacterial suspensions were added to the cell cultures at a multiplicity of infection of approximately 100 bacteria per cell and centrifuged (1,000 *g* for 10 min) to promote close contact between the bacteria and host cells (Saliba et al. 2005). Control cultures were exposed to culture medium alone. After incubation for 1 h at 37ºC, cells were incubated with culture medium containing 300 μg/mL gentamicin for different lengths of time to reduce the bacterial challenge and to assess the host response at later time points (Saliba et al. 2005, Lins et al. 2010).

Detection of released vWF - To detect vWF released into the cell supernatant, cells cultured in 6-well plates (4.5 x 10^5 cells/well) were infected for 1 h, rinsed and incubated with gentamicin-containing culture medium for 72 h (Ribeiro et al. 1995). The conditioned media from control and infected cultures were then concentrated 20-fold with a Speed Vac Plus (Savant) and the vWF concentration was assayed in duplicate by ELISA using purified rabbit anti-human vWF and a horseradish peroxidase-conjugated anti-vWF secondary antibody (Dako), as previously described (Bonnefoy et al. 2001). A standard curve was constructed from serial dilutions of normal pooled human plasma, assuming a vWF concentration of 1 unit/mL.

To detect the released vWF that was incorporated into the subendothelial matrix, cells were cultured in 96-well dishes (0.3 x 10^4 cells/well), infected for 1 h (or not infected) and treated with gentamicin-containing medium for 72 h. Cells were then washed with phosphate buffered saline (PBS) and treated for 20 min with PBS containing 0.1 M NH_4OH and 0.1% Triton X-100 to expose the native subendothelial matrix (Bonnefoy et al. 2001). After washing, the wells of the microtitre plate were fixed with 4% paraformaldehyde, treated with 1% bovine serum albumin (BSA) in PBS and exposed to the anti-vWF-horseradish peroxidase conjugate. The concentrations of vWF in the supernatant and the subendothelial matrix were normalised and reported in U/mL released by 10^5 cells.

Detection of vWF-expressing microparticles in cell supernatants - Cells were infected for 1 h and then treated with gentamicin-containing medium. Control cells were treated similarly with antibiotic-containing medium. After incubation at 37ºC for 19 h, the incubation media were cleared of cell debris by centrifugation (5,000 *g* for 15 min) and then centrifuged at 17,500 *g* for 30 min at 15ºC (Plotkowski et al. 2008). Sedimented microparticles were washed and treated for 30 min at room temperature (RT) with annexin V-Alexa Fluor 647 complexes (Invitrogen) to identify surface phosphatidylserine residues. After washing, microparticles were suspended in PBS containing 1% BSA and analysed by flow cytometry. The region corresponding to shed microparticles was defined in side-angle light scatter vs. fluorescent intensity dot plot representations using 0.5 μm and 0.9 μm fluorescent beads (Megamix) as a reference, according to the manufacturer's instructions. Because ExoU cytotoxicity leads to the partial death of PA103-infected cultured cells, the numbers of viable cells in the PA103 and PA103Δ*exoU*-infected culture wells and in the control uninfected culture wells were determined in parallel after the 1 h infection period. The microparticle concentrations detected in the cell culture supernatants were then normalised and reported as microparticles shed by 10^5 cells. In other assays, before treatment with the annexin V complex, sedimented microparticles were incubated with rabbit anti-human vWF for 1 h and then incubated for 30 min at RT with goat anti-rabbit IgG-FITC. After washing, microparticles were analysed by flow cytometry. The results were expressed as a "vWF-expression index" as described (Leytin et al. 2000) and this value was obtained by the following formula: percentage of vWF-expressing microparticles x median microparticle fluorescence intensity.

Detection of cell-associated vWF - Two different approaches were used to determine whether any portion of the secreted vWF remained associated with the cultured cells: flow cytometry and confocal microscopy analysis of nonpermeabilized cells. For the flow cytometry analysis, cells were infected for 1 h, treated with antibiotic-containing medium for an additional 5 h, washed with PBS to remove all microparticles that were associated with cell membranes, detached from the wells by trypsinisation and fixed in 4% paraformaldehyde in PBS containing 4% sucrose for 20 min. After washing with PBS, cells were treated with a rabbit anti-human vWF antibody for 1 h at 4ºC, washed and incubated with a goat anti-rabbit IgG-FITC complex (Santa Cruz Biotechnology). The cells were then washed again and suspended in PBS containing 1% BSA. At least 5,000 cells were analysed by flow cytometry. Control uninfected cells were processed similarly. The results were expressed as a "vWF-expression index", calculated as described above.

For the confocal microscopy analysis, cells were grown on glass coverslips, infected (or not infected) for 1 h, treated with antibiotic-containing medium for additional 5 h, washed to remove microparticles associated with cell

membranes, fixed with 4% paraformaldehyde and stained with anti-vWF antibody and goat anti-rabbit IgG-Cy3 complex (Zymed). In some assays, cells were subsequently stained with phalloidin-FITC (Sigma-Aldrich), washed and incubated with Topro (Invitrogen) for 5 min. Coverslips were mounted on glass slides and observed under a Zeiss LSM 510 Meta confocal microscope.

Detection of vWF mRNA by reverse transcription-polymerase chain reaction (RT-PCR) - Total RNA was isolated from uninfected (control) HMEC-1 cells or cells infected for 1 or 3 h using the Qiagen RNeasy kit. cDNA was synthesised from the total RNA by RT with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). The isolated cDNA was subjected to PCR under the following conditions: denaturation at 95ºC for 2 min, 33 (vWF) or 23 (ß-actin) cycles of denaturation at 95ºC for 45 sec, annealing at 50ºC for 45 sec and extension at 72ºC for 45 sec, and an additional extension step of 5 min at 72ºC after the last cycle. The primers used in the reactions were 5'-AGTTCATGGAGGAGGTGATTCAGC-3' (vWF sense), 5'-AGCCATCCAGGAGAAGGATCACG-3' (vWF antisense), 5'-CCTCGCCTTTGCCGATCC-3' (ß-actin sense) and 5'-GGATCTTCATGAGGTAGTCAGTC-3' (ß-actin antisense). PCR products were subjected to electrophoresis in a 1% agarose gel and densitometry was performed using Lab Image software (Kaplan GmbH, Germany).

Platelet adhesion to P. aeruginosa-infected cells - To investigate the relationship between increased vWF release by PA103-infected cells and enhanced platelet adhesion, cells cultured on glass coverslips were infected for 1 h and then treated with gentamicin-containing culture medium for additional 5 h. In parallel, human blood was collected in ACD anticoagulant solution (130 mM citric acid, 153 mM citrate trisodium and 111 mM glucose; pH 6.5) containing PGE_1 at 1 U/mL and centrifuged at 250 *g* for 8 min. The resulting platelet-rich plasma was centrifuged at 1,500 *g* for 10 min. Sedimented platelets were washed in platelet buffer (36 mM citric acid, 5 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 103 mM NaCl; pH 6.4) and resuspended at a concentration of 1.0 x 10^7 platelets/mL. Infected and control uninfected endothelial cells were then incubated with the platelet suspension for 30 min at RT. In other assays, platelet suspensions were perfused over endothelial cells for 5 min (10 mL/min) with a peristaltic pump (Pump-1, Pharmacia Biotech). Cells were then washed, fixed for 20 min with 4% paraformaldehyde in PBS containing 4% sucrose and stained with May-Grunwald-Giemsa stain. Coverslips were mounted on glass slides and examined with a Zeiss Axioplan optical microscope. The percentage of cells with adherent platelets present in at least 10 different microscopic fields was determined in two different assays, both of which were carried out in duplicate.

Statistical analysis - The results were expressed as the means ± standard error of the means of data obtained in at least three different experiments. Significant differences between groups were determined with one-way analysis of variance followed by Dunnett's or Bonferroni's multiple comparisons post-test. Statistical significance was set at a p value of < 0.05.

RESULTS

ExoU enhanced the release of vWF and vWF-bearing microparticles from microvascular endothelial cells - As shown in Fig. 1, the concentrations of vWF in both the cell culture supernatant (Fig. 1A) and subendothelial matrix (Fig. 1B) of endothelial cells infected with the ExoU-producing bacterial strain were significantly higher than those in control cultures or in cultures infected with the *exoU*-deficient mutant.

ExoU similarly enhanced the release of microparticles containing anionic phosphatidylserine in their exoplasmic leaflet, as revealed by their reactivity with the annexin V-FITC complex (Fig. 2B, D). More importantly, the vWF expression indices for microparticles shed after PA103 infection were significantly higher than those for microparticles shed by control cells or by cells infected with the PA103Δ*exoU* mutant (Fig. 2C, E).

PA103-infected cells exhibited increased vWF expression - Studies have shown that vWF multimers released from endothelial cells are anchored to the endothelial surface in the form of structures that are capable of binding platelets before being cleaved by plasma metalloproteases

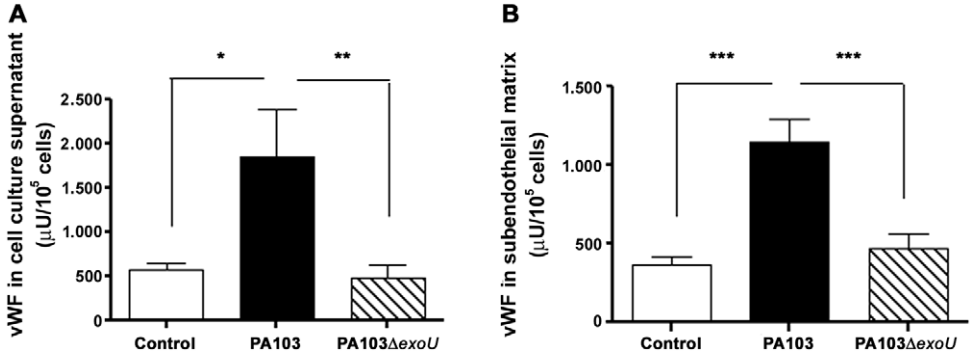


Fig. 1: concentration of von Willebrand factor (vWF) in cell culture supernatant (A) and subendothelial matrix (B) from control uninfected and infected endothelial cells detected by ELISA analysis. Data represent means and standard error of the means of the results from five assays carried out in duplicate (A) or eight assays carried out in quadruplicate (B). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

(Dong et al. 2002). To investigate whether PA103-infected cells harbour increased amounts of surface-associated vWF, nonpermeabilized control and infected cells were treated with an anti-vWF antibody and submitted to flow cytometry and immunocytochemistry analyses.

As shown in Fig. 3, the vWF-expression index of cells infected with the wild-type bacteria was significantly higher than those of uninfected control cells and cells infected with the mutant bacteria. Confocal microscopy analyses confirmed the increased expression of vWF on the surface of PA103-infected cells (Fig. 4).

Because ExoU is a phospholipase, we wondered whether the increased staining of PA103-infected cells with the anti-vWF antibody, detected by both flow cytometry and confocal microscopy, may have been caused by the permeabilization of cell membranes by the toxin; this permeabilization would enable the anti-vWF antibody to penetrate infected cells and stain vWF stored in Weibel Palade bodies. To address this question, nonpermeabilized fixed control and infected cells were treated with phalloidin-FITC, which labels cytoplasmic actin and with the anti-vWF antibody and then examined by confocal microscopy. Control and infected cells were equally poorly labelled with phalloidin-FITC (data not shown), thereby ruling out the false-positive staining of PA103-infected cells.

ExoU did not modulate the level of VWF mRNA expressed by infected cells - To determine whether the increased vWF expression and release from PA103-infected cells depended on the modulation of vWF mRNA

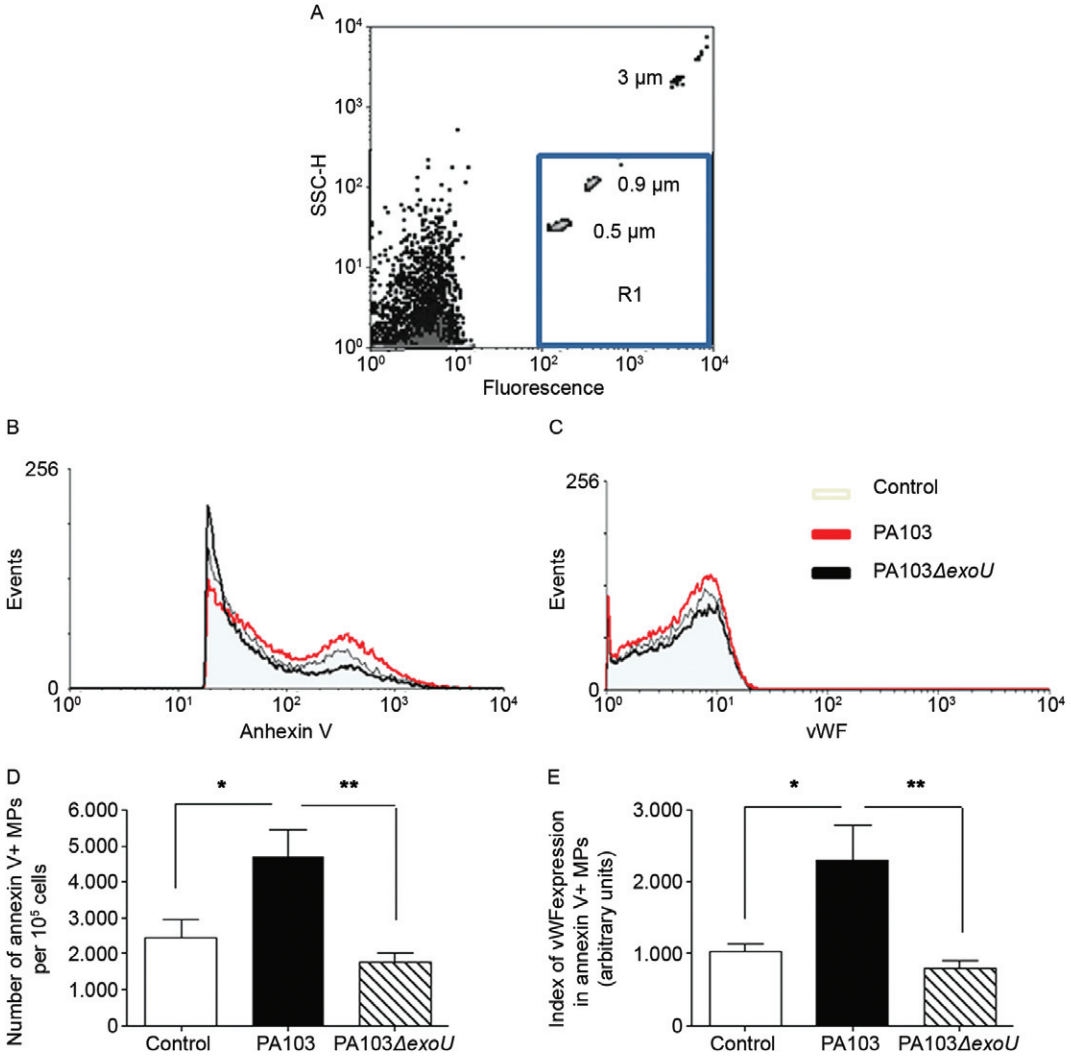


Fig. 2: release of microparticles (MPs) by control and infected endothelial cells detected by flow cytometry analysis. A: dot plot histogram showing the distribution of fluorescent beads used for the flow cytometer setting before analysis of MPs in region defined as R1; B: representative histogram showing increased number of annexin V-labelled MPs released by PA103-infected cells; C: representative histogram showing increased number of annexin V positive MPs expressing von Willebrand factor (vWF) released by PA103-infected cells; D: mean number and standard error of the means (SEM) of MPs released by 10^5 cells, as determined in three assays carried out in triplicate; E: index of vWF expression by annexin V positive MPs, obtained with the formula: percentage of vWF-expressing MPs x median of MP fluorescence intensity. Data represent means and SEM of the results from three assays carried out in triplicate. SSC-H: side scatter complexity histogram; *: $p < 0,05$; **: $p < 0.01$.

levels, we performed RT-PCR assays. No difference in mRNA levels was detected between control and *P. aeruginosa*-infected cells (data not shown).

ExoU enhanced platelet adhesion to infected endothelial cells - To evaluate whether the ExoU-induced release of vWF favoured platelet adhesion to endothelial cells, platelet suspensions were either incubated with or perfused over HMEC-1 cells cultured on glass coverslips. In PA103-infected cultures incubated with platelets under static conditions, the percentage of cells with adherent platelets was significantly higher than that in control cultures or in cultures infected with the *exoU* mutant (Fig. 5A). More interestingly, PA103-infected cultures exposed to platelets under flow conditions for only 5 min also exhibited a significantly higher percentage of cells with adherent platelets (Fig. 5B). No significant difference in the percentage of cells with adherent platelets was observed between cultures infected with the bacterial mutant and control cultures.

DISCUSSION

Endothelial cells usually provide an ideal surface for blood flow by inhibiting platelet adhesion and the initiation of blood clotting. In contrast, platelets adhere avidly to activated or injured endothelium via several adhesion molecules that are expressed on these cells (Wu & Thiagarajan 1996, Varga-Szabo et al. 2008). Because *P. aeruginosa* ExoU may directly damage endothelial cells (Saliba et al. 2006), we investigated whether this toxin might alter the thromboresistant phenotype of the microvascular endothelium and promote platelet adhesion.

We demonstrated that ExoU enhanced the release of vWF into the endothelial cell culture supernatant and subendothelial matrix. One explanation for this phenomenon might be that arachidonic acid released by PA103-infected cells, as previously reported by our group (Saliba et al. 2005), enhances the fusion of Weibel Palade bodies with the endothelial plasma membrane, resulting in vWF exocytosis (Connell et al. 2007, Darios et al. 2007). A second potential explanation is that vWF release may have resulted from ExoU-induced endothelial cell lysis. Because a similar injury of endothelial cells is likely to occur in vivo, the vWF release reported here is likely relevant; the immobilisation of vWF released towards the lumen of blood vessels on exposed subendothelial matrix is considered to be the predominant mechanism that initiates platelet response in areas of vascular injury (Ruggeri 2003).

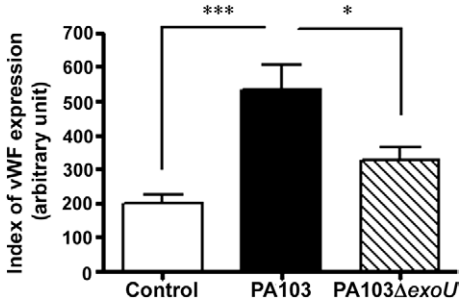


Fig. 3: index of cell-associated von Willebrand factor (vWF) in control uninfected and infected cells, obtained with the formula: percentage of vWF-expressing cells x median of cell fluorescence intensity. Data represent means and standard error of the means of the results from four assays carried out in triplicate. *: $p < 0,05$; ***: $p < 0.001$.

A proposed model for the regulation of vWF release is that upon stimulation, ultra-large vWF multimers are released and immediately anchored to the surface of endothelial cells via binding to P-selectin. These multimers are then stretched by fluid shear stress into an open conformation and cleaved by the metalloproteinase ADAMTS-13 into fragments of various sizes that are detected in the plasma of healthy individuals (Dong et al. 2002, Padilla et al. 2004). Because cultured endothelial cells are not exposed to fluid shear, uncleaved ultra-large vWF is likely to remain anchored to cell membranes. Such anchorage would explain both the increased expression of surface-associated vWF, as detected by confocal microscopy and the increased adhesion of non-stimulated platelets to PA103-infected cells.

The physiological relevance of vWF-mediated platelet adhesion to endothelial cells, rather than to sites of endothelial injury, has been highlighted by in vivo studies showing that resting platelets adhere to intact mouse mesenteric venules treated with a secretagogue of Weibel Palade bodies through a vWF-dependent and P-selectin-independent mechanism (Sporn et al. 1986, Andre et al. 2000). Although platelets recruited to the endothelium may release proinflammatory cytokines and modulate leukocyte function, thereby contributing to local defence mechanisms, they can also initiate deleterious thrombotic events.

Another finding from our study that may have in vivo relevance is the enhanced release of vWF-expressing microparticles from PA103-infected cells. Microparticles released from activated or apoptotic endothelial cells participate in platelet aggregation owing to the presence of functional adhesive glycoproteins in the plasma membranes. These glycoproteins bind to the A1 domain of immobilised and soluble vWF (Reininger 2008). In addition, high percentages of endothelial-derived microparticles (EMP) express ultra-large vWF multimers (Jimenez et al. 2003, Jy et al. 2005, Othman et al. 2007). The multimeric size of vWF is important for its function because lower molecular weight multimers show less binding to platelets and to collagen (Doucet-de Bruine et al. 1978, Santoro et al. 1983). In a study on the interaction between EMP and platelets, Jy et al. (2005) highlighted the importance of the large molecular weight vWF expressed by EMP. In the presence of ristocetin, EMP induced the aggregation of up to 95% of platelets and EMP-induced platelet aggregates were more stable than those induced by free soluble vWF. These results further support the procoagulant properties of endothelial microparticles.

In conclusion, this report describes the effects of endothelial cell infection with the ExoU-producing PA103 *P. aeruginosa,* showing that ExoU enhanced vWF release and that at least a portion of the released vWF remained associated with the cell surface. ExoU also enhanced the

release of prothrombotic vWF-bearing microparticles and platelet adhesion to infected cells. Although it is tempting to conclude that there is a cause-effect relationship between the increased expression of cell-associated vWF and increased platelet adhesion to PA103-infected cells, further studies are necessary to prove that vWF is involved in platelet-endothelial cell association.

In recent years, our laboratory has made progress in understanding the contribution of ExoU to the pathogenesis of *P. aeruginosa* infections. We first demonstrated that ExoU may induce a procoagulant activity in airway epithelial cells, resulting from the upregulated expression of TF and the increased release of tissue factor-positive microparticles (Plotkowski et al. 2008). More recently, Machado et al. (2010) demonstrated that ExoU can induce vascular hyper-permeability, platelet activation and thrombus formation in the lungs and renal microvasculature of mice with experimental *P. aeruginosa* pneumosepsis; these authors also observed an anti-fibrinolytic environment in mice airways secondary to the induction of enhanced plasminogen activator inhibitor-1 production (Machado et al. 2011). These results, as well as those from the present report, demonstrate the potential of ExoU to induce a prothrombotic state in host organisms.

ACKNOWLEDGEMENTS

To Maria Angélica P da Silva and Pedro Paulo de Abreu Manso, for her technical assistance, Dr Marcelo Santiago (Instituto de Biofísica Carlos Chagas Filho, UFRJ), for his support with confocal microscopy, and to PDTIS, for use of its facilities.

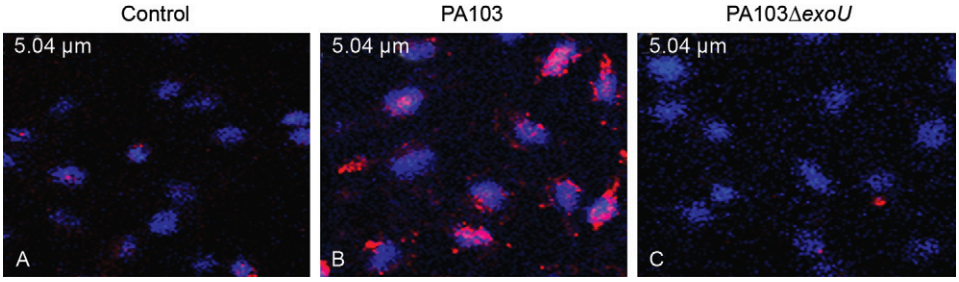


Fig. 4: confocal microscopy detection of von Willebrand factor (labelled in red) at the surface of nonpermeabilized paraformaldehyde-fixed control (A), PA103 (B) and PA103Δ*exoU*-infected endothelial cells (C). Cell nuclei appear labelled in blue.

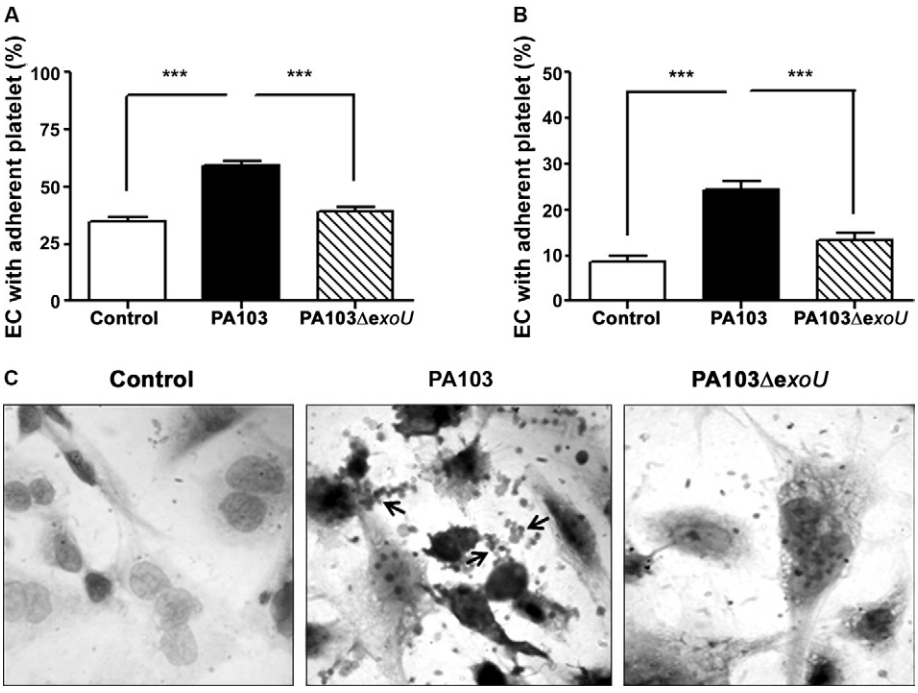


Fig. 5: platelet adhesion to control and infected endothelial cells. Percentage of cells with adherent platelets after cell incubation with platelet suspensions under static (A) and perfusion (B) conditions. Data represent means and standard error of the means from results of two experiments carried out in triplicate. C: light micrographs of control and infected endothelial cells exposed to platelet suspensions under static conditions. Arrows point to platelet aggregates. Asterisks mean $p < 0.001$.

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