Desmopressin Induces Endothelial P-Selectin Expression and Leukocyte Rolling in Postcapillary Venules

By Samina Kanwar, Richard C. Woodman, M.C. Poon, Toyoaki Murohara, Allan M. Lefer, Kelly L. Davenpeck, and Paul Kubes

Desmopressin, (DDAVP; 1-desamino-8-D-arginine vasopressin) increases the release and activity of von Willebrand factor (vWF); however, its effects on the other major constituent of endothelial Weibel-Palade bodies, P-selectin, has not been investigated. DDAVP-induced P-selectin expression may explain DDAVP's efficacy in bleeding disorders in which vWF levels are normal. Therefore, the objective of this study is to assess the effect of DDAVP on P-selectin expression on endothelial cells of postcapillary venules in vivo and on human umbilical vein endothelium in vitro, and to determine whether DDAVP has direct effects on leukocyte behavior in postcapillary venules. DDAVP (0.1 and 1.0 μg/mL) induced a significant but transient increase in P-selectin expression on human umbilical vein endothelial cells as well as on rat and human platelets. Immunohistochemical analysis of rat postcapillary venules showed that in contrast to saline, DDAVP injection (1 μg/kg, intravenous) induced significant endothelial P-selectin expression. DDAVP administration also induced a rapid and significant increase in leukocyte rolling in rat mesenteric venules in vivo. This response was entirely dependent on P-selectin, as an anti-P-selectin antibody rapidly reversed the DDAVP-induced increase in leukocyte rolling. DDAVP induced leukocyte rolling in medium (20 to 40 μm) and large (>40 μm), but not small (<20 μm), postcapillary venules. In animals that were treated with DDAVP, there was a steady and significant increase in leukocyte adhesion. This study shows that DDAVP can directly induce P-selectin expression on endothelium in vitro and in vivo and that the latter response is capable of supporting prolonged leukocyte rolling in rat postcapillary venules.

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For More Than a Decade Desmopressin (DDAVP; 1-desamino-8-D-arginine vasopressin), a synthetic derivative of the hormone L-arginine vasopressin, has been effectively used as a hemostatic agent in a variety of acquired and inherited bleeding disorders.1-4 DDAVP has been shown to rapidly shorten bleeding time and reduce blood loss in patients with mild and moderate hemophilia A, selected types of von Willebrand disease (vWD) and several forms of platelet dysfunction.5-8 In certain circumstances the need for blood products, and consequently the risk of transfusion-transmitted diseases, has been reduced because of DDAVP administration. Although a single unifying mechanism to explain the beneficial effects of DDAVP in the above heterogeneous group of disorders is lacking, there is considerable evidence to imply an improvement, albeit transient, in hemostasis after DDAVP administration.6,8 It has generally been assumed that the mechanism of action of DDAVP is to induce release of preformed high-molecular-weight multimers of von Willebrand factor (vWF) from the Weibel-Palade (WP) bodies of endothelial cells.9 Although increases in plasma vWF and factor VIII activity may explain the efficacy of DDAVP in patients with type 1 vWD and hemophilia A,1,5,8,10 the mechanism(s) in patients who have normal or elevated vWF remains unknown. Mechanisms independent of endothelial cell release of vWF have also been proposed.3,5,11-13

Besides vWF and its related propolypeptide, P-selectin is the only other constituent within WP bodies that is known to be expressed on endothelial cells and to have an adhesive function.14-16 Recent studies have shown that endothelial cell expression of P-selectin is important for the very early leukocyte-endothelial cell interaction known as leukocyte rolling.17-20 An absolute prerequisite for leukocyte adhesion and emigration.21-23 Although several agonists have been reported to mediate vWF release and P-selectin upregulation on endothelial cells,15,16,24 the effect of DDAVP on endothelial cell P-selectin expression and leukocyte-endothelial cell interactions has not been characterized. It is conceivable that DDAVP's efficacy in disorders other than type 1 vWD and hemophilia A may be partially caused by its ability to induce P-selectin expression and thereby mediate leukocyte-endothelial cell interactions.

Therefore, in this study we systematically examined the potential effect of DDAVP on P-selectin expression and function on endothelial cells of postcapillary venules. This was accomplished using immunohistochemical staining of rat mesenteric venules exposed to DDAVP and intravital microscopy to visualize leukocyte behavior in single postcapillary venules.

MATERIALS AND METHODS

Intravital microscopy preparation. Male Sprague-Dawley rats (180 to 250 g) were maintained on a purified laboratory diet and fasted for 24 hours before surgery. The animals were initially anesthetized with pentobarbital sodium (65 mg/kg body weight), and the right carotid artery and jugular vein were cannulated for systemic arterial blood pressure measurements (Statham P23A pressure transducer and a Grass physiologic recorder, Scarborough, Canada) and intravenous (IV) drug administration, respectively. A midline abdominal incision was made and a segment of the mid-jejunal mesent-
The cells were then fixed with 500 μL of 500 mM solution of DDAVP (0.1 or 1.0 μg/kg) and 96% humidity, for 24 hours, onto 48-well plates. Cultures were incubated in 5% CO2, at 37°C

Results were visualized and the following parameters were measured: venular diameter, leukocyte rolling and adhesion, leukocyte rolling velocity, centerline red blood cell (RBC) velocity, and shear rates. This preparation has been extensively used by us25-27 and others28,30 to examine leukocyte-endothelial cell interactions in vivo.

Experimental protocol. Immediately upon finding a postcapillary venule, the image was recorded for 5 minutes followed by three additional 5-minute recordings at 15, 30, and 60 minutes. For each set of experiments, all animals were pretreated before laparotomy with sodium cromoglycate (5 mg/kg IV) to minimize surgically induced leukocyte rolling as previously described.31 Animals received DDAVP in normal saline at 1 μg/kg as an IV bolus. In other animal models, investigators have used DDAVP ranging from 0.3 to 4.0 μg/kg body weight and have demonstrated enhanced hemodynamic and antidiuretic effects.32-34 The mesentery was continuously superfused with warmed bicarbonate-buffered saline, and leukocyte rolling and adhesion in the postcapillary venules were recorded for the remainder of the experiment.

To determine if the DDAVP-induced leukocyte rolling was mediated by P-selectin, another series of experiments was conducted. In these experiments, at 5 minutes of the experimental protocol, animals received DDAVP followed 15 minutes later by 2 mg/kg of an immunoneutralizing anti-P-selectin antibody, PB1.3 (P-selectin blocking IgGl-clone 352; Cytel Corp, San Diego, CA). Dose-response studies from our laboratory have previously shown that PB1.3 at 2 mg/kg completely inhibited P-selectin-dependent leukocyte rolling in vivo.35 Although medium-sized (20 to 40 μm in diameter) postcapillary venules are generally used to study leukocyte rolling in vivo,36 in a separate series of experiments we also examined the effect of DDAVP on leukocyte rolling in postcapillary venules of various diameters: small (<20 μm), medium (20 to 40 μm), and large (>40 μm).

P-selectin expression in vivo. Thirty minutes after DDAVP administration the superior mesenteric artery and vein were both cannulated for perfusion of the mesenteric circulation. The ileum was then perfused free of blood with modified Krebs-Henseleit buffer warmed to 37°C and bubbled with 95% O2 and 5% CO2. The tissue was subsequently fixed in 4% paraformaldehyde and immunohistochemical localization of P-selectin accomplished using a modified avidin-biotin immunoperoxidase technique as previously described.37 Thirty venules were examined and the percentage of positive staining venules determined. For control purposes similar surgical procedures were also performed on a group of sham operated animals that received only saline. We also measured concentrations of vWF in rat plasma before and after DDAVP administration using an enzyme-linked immunosorbent assay previously described.38

P-selectin expression on human umbilical vein endothelial cells (HUVEC) in vitro. Endothelial cells were isolated from human umbilical cord vessels as previously described.39 Briefly, umbilical cord veins were rinsed of formed blood elements with phosphate-buffered saline (PBS) containing penicillin (100 U/mL) and streptomycin (100 μg/mL). Collagenase (2.5 mg/mL; 149 U/mg) was instilled into the vein and the cord incubated for 20 minutes at 37°C and gently massaged to cause detachment of endothelial cells from the vessel wall. The digest was collected into centrifuge tubes, the collagenase inactivated with fetal calf serum, and centrifuged (400g for 10 minutes at 25°C). The pellet was resuspended in M199, without cell mitogens, containing 10% fetal calf serum and antibiotics plated onto 48-well plates. Cultures were incubated in 5% CO2, at 37°C and 96% humidity, for 24 hours.

The monolayers were then exposed to Hanks' buffered salt solution or a solution of DDAVP (0.1 or 1.0 μg/mL) for 2, 10, 20, or 60 minutes. The cells were then fixed with 500 μL of cold 1% formalin for 30 minutes at room temperature and then washed once with 500 μL PBS. The wells were blocked with 1 mL of PBS containing 1% bovine serum albumin (w/vol) and 0.01% NaN3 for 30 minutes followed by incubation with 100 μL of the primary antibody (S12) solution (10 μg/mL) for 30 minutes. This antibody was kindly provided by Dr R. McElver (University of Oklahoma). After three washes with 0.05% Tween 20 (Sigma Chemical Co, St Louis, MO)/PBS, 100 μL of the secondary antibody (peroxidase-labeled goat-antimouse IgG; Kirkegaard and Perry Laboratories Inc, Maryland) was added to the wells, incubated for 30 minutes, and washed three times. A substrate solution, ABTS peroxidase substrate (Kirkegaard and Perry Laboratories Inc) was then added to the wells and the reaction allowed to develop for 30 minutes. Absorbance was then measured at 405 nm.

Flow cytometric analysis of P-selectin expression on platelets. Flow cytometric analysis of P-selectin expression on rat and human platelets was performed by a method previously described.39 In brief, blood was collected from six male Sprague-Dawley rats and was anticoagulated with sodium citrate phosphate buffer (Sigma Chemical Co, St Louis, MO). Human blood was obtained from healthy adult volunteers. Platelet-rich plasma (PRP) was obtained and centrifuged to form a platelet-rich pellet. The pellet was washed and resuspended in Dulbecco’s PBS. Aliquots of the platelet suspensions were either incubated with buffer alone (unstimulated) or stimulated with hydrogen peroxide (H2O2; 100 μmol/L), a P-selectin inducer, or DDAVP (0.1 μg/mL) at 37°C for 15 minutes without stirring. The platelet suspensions were then treated with human blocking IgG (4.0 mg/mL; Sigma Chemical Co) and then the primary anti-P-selectin antibody, PB1.3 (20 μg/mL; Cytel Corp) was added and maintained at 4°C for 60 minutes. The platelets were then washed and F(ab')2, fragments of a goat-antimouse IgG-phycocerythrin conjugate (Tago, Burlingame, CA) were used as secondary antibody, and the cells analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA).

Statistical analysis. Data were analyzed using an analysis of variance and the Student’s t-test with a bonferroni correction for multiple comparisons. All values are expressed as means ± SE with significance at P < .05.

RESULTS

Table 1 summarizes the baseline hemodynamic parameters in animals before (0 minutes) and after (60 minutes) DDAVP administration. There were no significant differences in RBC velocity, venular diameter, and shear rates over time with DDAVP treatment. These data are consistent with the observations that DDAVP acts through a V1 (vasoressin)-receptor on endothelium, rather than a V1-receptor, which is found primarily on smooth muscle cells and causes contraction.38 Table 1 also shows that DDAVP did not induce changes in leukocyte rolling velocity.

DDAVP administration at 5 minutes induced a significant increase in the flux of rolling leukocytes that was sustained
Fig 1. DDAVP administration at 5 minutes of the experimental protocol induced a significant increase in the flux of rolling leukocytes in medium venules (20 to 40 μm). This increase in leukocyte rolling was maintained for the entire duration of the experiment (60 minutes), n = 6. *P < .05 relative to time 0 minutes.

Fig 2. This figure shows the induction of leukocyte rolling in venules of various sizes. DDAVP induces a significant increase in leukocyte rolling in large postcapillary venules (>40 μm in diameter, n = 8), but has no effect on leukocyte rolling in small venules (<20 μm, n = 4). *P < .05 relative to 0 minutes.

Fig 3. This figure shows the number of adherent leukocytes within a given segment of medium-sized (20 to 40 μm in diameter) postcapillary venules. DDAVP administered at 5 minutes induces a subtle but significant increase in leukocyte adhesion at 60 minutes, n = 6. *P < .05 relative to time 0 minutes value. †P < .05 relative to respective control value.

Fig 4. DDAVP increases leukocyte rolling via P-selectin. DDAVP administration at 5 minutes induces a significant increase in leukocyte rolling in medium sized vessels (20 to 40 μm in diameter). An anti-P-selectin antibody, PB1.3, administered at 20 minutes rapidly reverses the DDAVP-induced increase in leukocyte rolling, n = 4. *P < .05 relative to time 0 minutes. †P < .05 relative to time 15 minutes.

for the entire duration of the experiment (Fig 1). The vessels used in this experiment ranged in diameter from 20 to 40 μm (medium size). DDAVP also induced a significant and sustained increase in leukocyte rolling in relatively large (>40 μm) venules (Fig 2). The peak increase in leukocyte rolling flux was similar in these vessels as in the medium-sized (20 to 40 μm) venules shown in Fig 1. However, DDAVP treatment did not support leukocyte rolling in postcapillary venules smaller than 20 μm (Fig 2).

The number of adherent leukocytes per 100 μm venule length in medium-sized vessels in control and DDAVP-treated animals is illustrated in Fig 3. In contrast to controls, animals that received DDAVP had a steady, time-dependent increase in leukocyte adhesion that reached significance at 60 minutes. It should be noted that the increase in adhesion in some venules was as high as 22 cells/100 μm length of venules. Comparatively, leukocyte adhesion with pro-inflammatory agents such as platelet-activating factor and leukotriene B₄ is approximately 15 to 30 cells/100 μm length venule, suggesting a reasonably profound increase in leukocyte adhesion with DDAVP.

Figure 4 shows that DDAVP induced a rapid increase in leukocyte rolling flux that could be rapidly and completely...
Fig 5. Immunohistochemical analysis of P-selectin expression in vivo in DDAVP-treated (A) and control (B) rats. DDAVP significantly increased P-selectin expression in medium-sized postcapillary venules compared with similar-sized venules from control rats.

reversed with an anti-P-selectin antibody, PB1.3. This PB1.3-associated reduction in leukocyte rolling persisted for the remainder of the experiment. To confirm that DDAVP was inducing WP body mobilization and P-selectin expression, immunohistochemical analysis of P-selectin expression and plasma levels of vWF were measured in untreated and DDAVP-treated animals. In animals treated with DDAVP, 51.2% ± 7.3% of postcapillary venules were strongly positive for P-selectin staining. This is in contrast to tissue sections from animals treated with saline alone in which only 13.4% ± 2.3% of venules stained positive for P-selectin. Virtually no P-selectin expression was observed in arterial endothelium or in postcapillary venules smaller than 20 μm in diameter (data not included) from either group of animals. Figure 5 shows P-selectin expression in DDAVP-treated (A) and control (B) mesenteric preparations. Plasma concentration of vWF was significantly increased after DDAVP administration from 0.39 ± 0.03 to 0.62 ± 0.1 U/mL (n = 5).

To determine whether DDAVP induced P-selectin expression on human endothelium, we exposed HUVEC to DDAVP. In contrast to untreated HUVEC, incubation of HUVEC with DDAVP (0.1 and 1.0 μg/mL) increased P-selectin expression within 2 minutes (Fig 6). This response was maintained for at least 20 minutes. Interestingly, at 60 minutes the DDAVP (0.1 or 1.0 μg/mL) induced P-selectin expression had returned to baseline. This transient P-selectin expression in vitro is consistent with results of endothelial cell P-selectin expression induced by other agonists, including thrombin and histamine. It should be noted that DDAVP did not induce P-selectin expression on passaged endothelial cell cultures or primary cultures that received a cocktail of growth mitogens (Endothelial Mitogen obtained from Biomedical Technologies, Inc, Stoughton, MA) overnight (data not shown).

To confirm that DDAVP also affected platelets, we measured P-selectin expression on rat and human platelets. Table 2 shows the percent positive staining for P-selectin on unstimulated platelets and platelets stimulated with H2O2 or DDAVP. The anti-P-selectin antibody bound to only a small percentage of rat and human platelets. However, after incubation with H2O2 and DDAVP the binding of the anti-P-selectin antibody to both the rat and human platelet surface was significantly increased, suggesting that both H2O2 and DDAVP significantly increase platelet P-selectin expression.
have been attributed to the release of high-molecular-weight WP bodies. Increased plasma levels of vWF and factor VIII would certainly explain the efficacy of DDAVP in type II vWD and hemophilia A; however, in the other DDAVP-responsive bleeding disorders, associated with normal or elevated plasma levels of vWF, the mechanism of action of DDAVP remains unknown. It has been suggested that DDAVP may have additional mechanism(s) of action irrespective of DDAVP's ability to increase plasma vWF. One potential consideration is a direct and local effect of DDAVP on the endothelium inasmuch as DDAVP also directly caused P-selectin expression on cultured endothelial cells. The effects of DDAVP on endothelial P-selectin expression in vivo has, to our knowledge, not been previously reported. It might be predicted that because P-selectin is stored within WP bodies of endothelial cells, DDAVP would influence P-selectin expression in a manner similar to other endothelial cell secretagogues. Thrombin, calcium ionophore, complement components C5b-9, phorbol esters, and histamine have all been previously reported to increase P-selectin expression simultaneously with the release of vWF. Stimulation with these agonists leads to a rapid exocytosis of WP bodies releasing preformed vWF multimers and exposing P-selectin on the surface of the plasma membrane.  

**DISCUSSION**

Commonly the beneficial hemostatic effects of DDAVP have been attributed to the release of high-molecular-weight vWF multimers from endothelial cells due to exocytosis of WP bodies. Increased plasma levels of vWF and factor VIII would certainly explain the efficacy of DDAVP in type II vWD and hemophilia A; however, in the other DDAVP-responsive bleeding disorders, associated with normal or elevated plasma levels of vWF, the mechanism of action of DDAVP remains unknown. It has been suggested that DDAVP may have additional mechanism(s) of action irrespective of DDAVP's ability to increase plasma vWF. One potential consideration is a direct and local effect of DDAVP on the endothelium inasmuch as DDAVP also directly caused P-selectin expression on cultured endothelial cells. The effects of DDAVP on endothelial P-selectin expression in vivo has, to our knowledge, not been previously reported. It might be predicted that because P-selectin is stored within WP bodies of endothelial cells, DDAVP would influence P-selectin expression in a manner similar to other endothelial cell secretagogues. Thrombin, calcium ionophore, complement components C5b-9, phorbol esters, and histamine have all been previously reported to increase P-selectin expression simultaneously with the release of vWF. Stimulation with these agonists leads to a rapid exocytosis of WP bodies releasing preformed vWF multimers and exposing P-selectin on the surface of the plasma membrane. P-selectin upregulation occurred within 5 minutes and persisted for nearly 30 minutes before undergoing re-endocytosis. Our results with DDAVP showed a similar time course of endothelial P-selectin expression in vitro, but a more prolonged time course in vivo. We have also shown for the first time that DDAVP has a direct effect on rat and human platelet P-selectin expression in vitro. The functional significance of DDAVP-induced platelet P-selectin expression remains to be characterized.

In addition to the increased P-selectin expression in vivo, there was a profound increase in leukocyte rolling after DDAVP administration that persisted for the entire duration of the experiment (60 minutes). These two events were causally related as an anti-P-selectin antibody completely reversed the DDAVP-induced increase in leukocyte rolling. These observations suggest that DDAVP directly stimulates vascular endothelial cells to express P-selectin, which is functionally manifested as increased leukocyte rolling in postcapillary venules. The increase in leukocyte rolling was evident in medium and large postcapillary venules, but not in small venules. Although it is tempting to conclude from this functional data that P-selectin expression was invoked only in vessels larger than 20 μm, it is also possible that in these very small vessels wherein the diameter of the leukocyte approaches that of the vessel lumen, recruitment of additional rolling cells may be less likely to occur.

Although this is the first documentation that DDAVP directly affects leukocyte rolling via P-selectin expression, there is ample evidence both in vitro and in vivo to implicate a role for P-selectin as an important adhesion molecule involved in leukocyte rolling. Reconstitution of P-selectin in lipid bilayers or activation of the endothelium with histamine has been shown to support leukocyte rolling under shear conditions in flow chambers. More recently, Kubes and Kanwar and Asako et al. showed that histamine administration can support prolonged (60 minutes), P-selectin-dependent leukocyte rolling in postcapillary venules. DDAVP induced a very similar response in vivo to that reported for histamine in that the P-selectin-dependent leukocyte rolling persisted for 60 minutes despite the fact that in vitro, DDAVP, as well as histamine, only induced transient P-selectin expression (<60 minutes). A possible explanation for this difference may be that in vitro endothelium isolated from large veins responds differently than the postcapillary venular endothelium, which is the normal site of leukocyte rolling in vivo.

It is interesting that DDAVP has previously been reported not to mobilize WP bodies to the surface of endothelial cells in vivo. However, in those studies, passaged endothelium

### Table 2. Flow Cytometric Analysis of P-Selectin Expression

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<th>Rat Platelets</th>
<th>Human Platelets</th>
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<td>% Positive</td>
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<td>Cells</td>
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<tr>
<td>Unstimulated</td>
<td>14 ± 6</td>
<td>23 ± 9</td>
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<tr>
<td>H2O2 (100 μmol/L)</td>
<td>78 ± 5*</td>
<td>63 ± 4*</td>
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<td>DDAVP (0.1 μg/mL)</td>
<td>45 ± 21*</td>
<td>58 ± 14†</td>
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* P < .01 relative to unstimulated group.
† P < .05 relative to unstimulated group.
was used whereas herein we used primary cultures of endothelium. Indeed, DDAVP had no effect in our system on passaged endothelium or on primary cultures wherein growth factors were added (data not shown). The need for primary cultures to induce P-selectin expression is consistent with observations by other investigators and highlights the very specific conditions required to mimic the in vivo situation. Collins et al. have reported that trypsin (commonly used to passage endothelium) affects WP body mobilization, perhaps explaining the lack of effect of DDAVP on P-selectin expression on passaged endothelial cells.

The results from our studies have several important implications. First, they support the growing body of evidence for the assigned role of P-selectin mobilized from WP bodies in mediating leukocyte rolling.6-20 Second, DDAVP’s ability to enhance leukocyte-endothelial cell interactions raises the question as to whether DDAVP’s efficacy in disorders other than type I vWD and hemophilia A (in which DDAVP’s mechanism of action is through the release of endothelial vWF) is through a direct effect on endothelial cells to enhance P-selectin expression. Whether this DDAVP-induced endothelial cell P-selectin expression is associated with improved platelet–endothelial interactions remains to be characterized. The clinical consequences and applications of these novel observations are incompletely understood but merit further investigation.

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