Desmopressin Induces Adhesion of Normal Human Erythrocytes to the Endothelial Surface of a Perfused Microvascular Preparation

By Han-Mou Tsai, Ira I. Sussman, Ronald L. Nagel, and Dhananjaya K. Kaul

The interaction of red blood cells (RBCs) with vascular endothelium under flow conditions was investigated using the perfused rat mesocecum. Under videomicroscopy, normal human erythrocytes were found to adhere to the venular endothelium of desmopressin-treated microvasculature. Transmission electron microscopy showed that the erythrocytes were attached to the endothelial cells at discrete electron-dense sites. Compared with control preparations in which the microvasculature was perfused with Ringer's-albumin solution alone, more than a 10-fold increase in radioactivity was retained in the desmopressin-treated microvasculature when technetium (99mTc)-labeled erythrocytes were infused into the vasculature. This erythrocyte adherence was accompanied by a higher increment in vascular resistance during the passage of RBCs through the microcirculation, and by a delay in the recovery toward baseline. The erythrocyte adherence in desmopressin-treated microvasculature was completely abolished with antibodies to von Willebrand factor (vWF). Desmopressin infusion in rats resulted in elevated vWF antigen levels and the appearance of extra-large molecular weight forms of vWF in plasma. These findings suggest that normal erythrocytes adhere to desmopressin-conditioned microvascular endothelium and that endothelial cell-derived vWF is involved in the erythrocyte-endothelium interaction.

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R ECENT STUDIES have explored the interaction of abnormal red blood cells (RBCs) with endothelium, but significant adherence of normal RBCs to vascular surfaces has not been reported. Sickle erythrocytes have been found to adhere to cultured human umbilical vein endothelial monolayers, and the endothelial surface of isolated rat mesocecum microvasculature. In these studies, normal RBCs exhibited no appreciable adherence. Although the mechanisms of this adherence remains unexplained, extra-large molecular weight (mol wt) forms of von Willebrand factor (vWF) derived from endothelial cells have been reported to enhance the adherence of sickle cells to cultured human umbilical vein endothelial monolayers.

1-Deamino-8-D-arginine vasopressin (desmopressin or DDAVP), a derivative of arginine vasopressin, causes in vivo release of vWF including extra-large mol wt forms. Similar large forms are released from cultured endothelial cells. We have used desmopressin to investigate the interaction of normal RBCs with endothelial surfaces when these cells are perfused through an ex vivo microvascular preparation. We find that in the desmopressin-treated microvasculature, normal RBCs exhibit abundant adherence to the venular endothelium. This adherence is mediated by endothelial cell vWF, since it is blocked by antibodies against vWF.

MATERIALS AND METHODS

RBCs: Blood was drawn from normal human donors into heparin Vacutainer tubes (Becton Dickinson, Rutherford, NJ) and centrifuged at 2,000 rpm for 2 minutes. The plasma and buffy coat were removed, and RBCs were washed three times in 0.9% saline solution, once in Ringer's solution (154.3 mmol/L NaCl, 5.63 mmol/L KCl, 2.16 mmol/L CaCl₂, and 0.6 mmol/L MgCl₂) containing 0.5% bovine serum albumin (BSA), and suspended in Ringer's-BSA to a hematocrit of 30%.

Preparation and perfusion of rat mesocecum microvasculature. The procedures have been previously described in detail. Briefly, the denervated mesocecum of anesthetized rats was isolated by cannulation of the ilio-colic artery and vein. The arterial perfusion pressure (Pa) and the venous flow rate (Fv) were constantly monitored and recorded on a polygraphic recorder throughout the perfusion. Flow of RBCs through the microvasculature was observed and recorded using a microscope-television camera-video recorder system. In control experiments, after a period of baseline perfusion (arterial perfusion pressure = 70 mm Hg) with Ringer's solution containing 2% bovine serum albumin (Ringer's-BSA), a bolus of RBCs (volume = 0.2 mL, Hct = 30%) was injected into the arterial line. In desmopressin experiments, the perfusion solution was switched to Ringer's-BSA containing 1.0 µg/mL desmopressin (DDAV injection; Ferring Pharmaceuticals, Malmo, Sweden). Perfusion was continued for 5 minutes until a steady state was established and then a 0.2 mL bolus of RBCs was injected into the arterial line during the perfusion with desmopressin in Ringer's-BSA. The venous effluents in both the control and desmopressin perfusions were collected and mixed 9 to 1 (vol/vol) with 3.2% sodium citrate. Cells were removed by centrifugation and the supernatants were stored at −70°C.

The calculated hemodynamic parameters were peripheral resistance unit (PRU) = arterial-venous pressure difference/venous outflow rate per gram of mesocecum, and time for pressure and flow to return to baseline (Tp). A transmission electron microscopy. The rat mesocecum was fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for 1 hour. Areas of the vasculature with large numbers of adherent and/or entrapped RBCs were readily identifiable under a stereoscopic microscope and were selected for processing for electron microscopy. After a 15 minute wash in 0.1 mol/L cacodylate buffer, the tissue was postfixed with 1% osmium tetroxide/0.1 mol/L cacodylate buffer (pH 7.4) for 1 hour followed by a buffer rinse for 15 minutes and a 5-minute rinse in distilled H₂O. The tissue was then transferred to 1% uranyl acetate for 1 hour, dehydrated in a graded series of ethanol solutions and embedded in EPON (Ladd Research, Burlington, VT). Sections (80 nm) were cut using an LKB III Ultramicrotome, stained with uranyl acetate, followed by lead citrate and viewed in a JEM 100 CX electron microscope (Japan Electronic Lab).

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Quantitation of erythrocyte trapping. For quantitation of erythrocyte trapping in the microvasculature, normal RBCs were labeled with technetium (99mTc) using the published methods. The radioiodinated RBCs were injected into the isolated rat mesococcum vascular preparations, as described above. Radioactivity retained in the vascular preparations was counted using a gamma counter (model 1282 compu gamma, LKB, Gaithersburg, MD). The volume of RBCs trapped per gram of tissue was determined by dividing the radioactivity per gram of tissue by the radioactivity per microliter of packed RBCs in the preinfusion sample.

Anti-vWF antibody in desmopressin perfusion. Rabbit antibody (immunoglobulin G [IgG] fraction) against human vWF and normal rabbit IgG were obtained from the Dako Corporation, Santa Barbara, CA. According to the manufacturer's package insert, traces of other antibodies have been removed by solid-phase absorption so that the antibody gives only one precipitation line in crossed immunoelectrophoresis against fresh human plasma. The specificity of the antibody for vWF was further demonstrated using immunoblotting technique. Purified human vWF, fibronectin and thrombospondin, and rat fibronectin were electrophoresed in SDS-polyacrylamide gel and electrically transferred onto Zeta-Probe membrane (Bio-Rad, Richmond, CA). The anti-vWF antibody that was bound to vWF was probed with alkaline phosphatase-conjugated goat anti-rabbit IgG and visualized with the substrate 5-bromo, chloro, 3-indoly phosphate/nitro blue tetrazolium (Bio-Rad). No binding of the anti-vWF antibody to fibronectin or thrombospondin was detected. Under similar conditions, rabbit anti-human fibronectin (Calbiochem, San Diego, CA) bound to both human and rat fibronectin.

Rat mesococcum was isolated and the vasculature was infused with desmopressin (1.0 μg/L) in Ringer's-BSA solution. After a 5-minute incubation at 25°C, the preparation was then infused with 5 mL Ringer's-BSA containing 200 μg/mL anti-vWF antibody, with Ringer's-BSA containing 200 μg/mL normal rabbit IgG, or with Ringer's-BSA alone, and incubated at 25°C for 30 minutes with the venous outflow cannula clamped. In separate experiments, the desmopressin-perfused vasculature was similarly incubated with anti-human fibronectin (Behring Diagnostics, La Jolla, CA) or with rabbit anti-human thrombospondin (courtesy of Dr Russel Howard, DNAX, Palo Alto, CA), each at a concentration of 200 μg/mL in 5 mL of Ringer's-BSA. Perfusion with Ringer's-BSA was resumed at 70 mm Hg and a bolus (0.2 mL) of normal RBCs was delivered into the arterial line. The perfusion pressure, venous outflow rate, and microscopic flow of RBCs were recorded as above.

Desmopressin infusion in rats. The change in rat plasma vWF after desmopressin infusion was studied using pentobarbitol-anesthetized rats weighing 150 to 250 gm. The femoral veins were cannulated for blood sampling and flushed with a small amount of heparin-saline solution after each sampling. Desmopressin, 0.4 to 16 μg/kg body weight, was diluted in 0.5 mL normal saline and infused slowly intraarterially or intravenously. Blood (0.45 mL) was drawn immediately before and at designated intervals after desmopressin infusion into polypropylene tubes containing 0.05 mL of 3.2% trisodium citrate. Platelet-poor plasma was immediately separated and stored at −70°C.

Multimeric analysis of rat vWF. Multimeric composition of rat vWF in plasma and perfusion effluents was analyzed by the method of SDS-glyoxal agarose gel electrophoresis as previously described. Plasma von Willebrand factor antigen levels. The vWF antigen (vWF:Ag) levels in rat plasma and perfusion effluents were determined with a solid-phase immunoradiometric assay similar to that described by Fricke. Briefly, 5 μL of serially diluted plasma samples were applied to 5 × 5 mm wells cut from 1% agarose gel poured on Gel-Bond film (FMC, Rockland, ME). The wells were then covered with the same agarose kept at 36°C, and the gel was fixed in 25% isopropanol–10% acetic acid solution, washed extens-
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sion was found in the postcapillary venules and smaller venules. In contrast, rare or no RBC adhesion was seen in the control experiments with Ringer’s-perfused microvasculature (Fig 1A). Electron microscopy confirmed the endothelial-RBC adhesion in desmopressin-perfused venules (Fig 2, A and B). The sites of RBC adhesion to the endothelium appeared distinct and electron-dense (Fig 2C).

Quantitation of RBC adhesion. In control experiments in which the vascular preparations were perfused with Ringer’s-BSA (n = 3), infusion of technetium-labeled RBCs resulted in retention of 0.08 to 0.16 μL of cells per gram of tissue (Table 1). In contrast, 0.54 to 1.9 μL of RBCs per gram of tissue were retained in the desmopressin-perfused vascular preparations (n = 3).

Hemodynamic measurements. As shown in Table 2, the changes in the peripheral resistance (PRU) associated with the passage of erythrocytes through the microcirculation, as well as the subsequent pressure-flow recovery time (Tpf), were consistent with the directly observed RBC adhesion. In the vascular preparations perfused with Ringer’s-BSA (n = 4), the RBC bolus caused slight increases in PRU, followed by complete pressure-flow recovery in a brief period of time (Tpf). When the vascular preparations were perfused with desmopressin in Ringer’s-BSA (n = 5), a slight increase in peripheral resistance was noticed, most likely due to a mild vasoconstrictive effect of desmopressin. Boluses of RBCs into the desmopressin-perfused vascular preparations resulted in higher PRU increments than those seen in the experiments using Ringer’s-BSA perfused preparations. The recovery of pressure and flow (Tpf) was also delayed.

Effect on RBC adhesion of anti-vWF. In the vascular preparations perfused with desmopressin followed by incubation with Ringer’s-BSA containing antibody to vWF (200 μg/mL, n = 3), boluses of RBCs resulted in slight increases in PRU followed by rapid recovery of pressure and flow (Table 2). These hemodynamic changes were comparable with those associated with AA RBC boluses in Ringer’s-BSA perfused preparations (Table 2, experiment 1). Microscopic observations showed no RBC adhesion in the anti-vWF experiments. On the other hand, in the experiments in which desmopressin-perfused vascular preparations were incubated with Ringer’s-BSA alone (n = 3) or with Ringer’s-BSA containing normal rabbit IgG (200 μg/mL, n = 3), the infusion of normal RBCs resulted in widespread RBC adhesion in the venules comparable with that seen in the desmopressin-perfused preparations (Table 2, experiment 1).

Fig 2. Transmission electron micrographs of venules showing normal RBC adhesion to the endothelial surface in desmopressin (1.0 μL/L in Ringer’s) perfused vasculature. (A and B) RBC (R) adhesion to the surface of an endothelial cell (E) (arrows pointing to attachment sites) during flow conditions. (C) Points of RBC attachment to the endothelial surface appear electron-dense. Bar = 0.5 μm.
Table 1. Quantitation of Technetium (99mTc)-Labeled Normal RBCs Trapped in the Microcirculation

<table>
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<tr>
<th>Exp No.*</th>
<th>Volume of Packed Cells Injected (µL)</th>
<th>µL Cells</th>
<th>Tissue Wt.</th>
<th>Trapped/µL Tissue WL</th>
<th>Ratio DDAVP/Control</th>
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<tbody>
<tr>
<td>Control (No DDAVP)</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>55.4</td>
<td>0.16</td>
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<td>-</td>
<td></td>
</tr>
<tr>
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<td>62.6</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>3</td>
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<td>0.12</td>
<td>-</td>
<td>-</td>
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<td>DDAVP</td>
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<td>1.90</td>
<td>15.8</td>
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</table>

*Six preparations were used. Aliquots from a given blood sample were infused in the absence or presence of DDAVP in separate preparations.

2b). Also, the changes in PRU and Tpf were similar to those observed in the desmopressin group.

In two separate experiments in which the desmopressin-perfused vasculature was incubated with either antibody to fibronectin (200 µg/mL) or anti-thrombospondin (anti-TSP) antibody (200 µg/mL), the infusion of AA cells resulted in significant adhesion, suggesting no effect of these antibodies. The PRU and Tpf changes were comparable with the desmopressin group (anti-fibronectin: PRU 24.2% increase, Tpf 50 seconds; anti-TSP: PRU 28.3%, Tpf 90 seconds).

Responses of plasma vWF to desmopressin. The results of plasma vWF:Ag levels after desmopressin infusion in rats are summarized in Fig 3. The response did not show dose-dependency for desmopressin doses of 0.4 µg/kg to 16.0 µg/kg. On average, the vWF:Ag levels at 15 and 30 minutes after desmopressin infusion were 58 ± 21% and 39 ± 18% respectively, above baseline levels. In the control infusions with normal saline, there were no changes in vWF:Ag levels, indicating that the responses were due to desmopressin and not anesthesia and/or surgical procedures. The multimeric composition of vWF in plasma as determined by the SDS-glyoxal agarose gel electrophoresis after desmopressin infusion in one rat is shown in Fig 4. However, vWF was not detected in the venous effluents of desmopressin-treated preparations.

DISCUSSION

Sickle and Plasmodium falciparum-infected erythrocytes have been reported to adhere both to cultured endothelial monolayers and postcapillary venules in the ex vivo...
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mesocecum vasculature under flow conditions. In the first case, vWF has been implicated, while thrombospondin might be involved in the malaria-infected RBC adhesion. Normal erythrocytes, on the other hand, did not adhere to the endothelial surface in any significant numbers in these experiments. However, the present-study demonstrated that normal erythrocytes may also adhere to vascular endothelium under certain conditions, such as treatment with desmopressin. The adhesion of RBCs in the desmopressin-treated rat mesocecum vasculature was clearly demonstrated both by light microscopy and increased retention of radiolabeled RBCs. Transmission electron microscopy showed the direct attachment of erythrocytes to endothelial cells at discrete, electron-dense sites. Erythrocyte adhesion under these conditions was hemodynamically significant, as demonstrated by the higher increment of vascular resistance and the delay in the subsequent recovery to baseline. However, no persistent blockage of the microcirculation was associated with the adhesion of normal erythrocytes. This is in contrast to findings with sickle and Plasmodium falciparum-infected erythrocytes, in which a significant microvascular vasocclusion was usually detected. Whether this is due to the difference in the magnitude of erythrocyte adhesion or differences in the hemorheologic properties of the erythrocytes involved cannot be determined.

Desmopressin causes elevation of vWF antigen levels and the appearance of extra-large forms of vWF in plasma when given to human subjects and to rats, as shown by the present data. Previous studies, as well as the present one, have failed to demonstrate the release of vWF from endothelial cells in isolated vascular preparations. However, the present data demonstrate that rat plasma vWF consists of a series of multimers and that extra-large forms of vWF appear in circulation as a result of desmopressin infusion into the whole animal. The data also indicate that endothelial vWF is involved in desmopressin-induced erythrocyte adhesion to vascular endothelium since the adhesion is abolished by antibodies to vWF. These findings suggest that endothelial vWF is expressed on the surface of the microvasculature as a result of desmopressin treatment and that the amount of vWF released into the perfusion solution is too low to be detected in the effluent.

Adhesion of normal erythrocytes was localized almost exclusively to venular rather than arteriolar surface. A similar distribution has also been observed with sickle erythrocytes, as well as with Plasmodium falciparum-infected RBCs. Whether this is due to the low-wall shear rates characteristic of venules, heterogeneous distribution of receptors to adhesive proteins, or heterogeneous distribution of vWF in different vascular beds (or a combination of the above) will have to be explored in the future.

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