Peptides based on αV-binding domains of erythrocyte ICAM-4 inhibit sickle red cell-endothelial interactions and vaso-occlusion in the microcirculation

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1Department of Medicine, Albert Einstein College of Medicine, Bronx, New York; 2Bristol Institute for Transfusion Sciences, National Blood Service, Bristol, United Kingdom; 3New York Blood Center, New York, New York; and 4Department of Life Sciences, University of California, Lawrence Berkeley National Laboratory, Berkeley, California

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Kaul, Dhananjay K., Xiao-du Liu, Xiaoxin Zhang, Tosti Mankelow, Stephen Parsons, Frances Spring, Xiuli An, Narla Mohandas, David Anstee, and Joel Anne Chasis. Peptides based on αV-binding domains of erythrocyte ICAM-4 inhibit sickle red cell-endothelial interactions and vaso-occlusion in the microcirculation. Am J Physiol Cell Physiol 291:C922–C930, 2006. First published June 7, 2006; doi:10.1152/ajpcell.00639.2005.—Growing evidence shows that adhesion molecules on sickle erythrocytes interact with vascular endothelium leading to vaso-occlusion. Erythrocyte intercellular molecule-4 (ICAM-4) binds αV-integrins, including αVβ3 on endothelial cells. To explore the contribution of ICAM-4 to vascular pathology of sickle cell disease, we tested the effects of synthetic peptides, V16PFVWRMS (FWV) and T91RWATSRI (ATSR), based on αV-binding domains of ICAM-4 and capable of inhibiting ICAM-4 and αV-binding in vitro. For these studies, we utilized an established ex vivo microvascular model system that enables intravital microscopy and quantitation of adhesion under shear flow. In this model, the use of platelet-activating factor, which causes endothelial oxidant generation and endothelial activation, mimicked physiological states known to occur in sickle cell disease. Infusion of sickle erythrocytes into platelet-activating factor-treated ex vivo rat mescecum vasculature produced pronounced adhesion of erythrocytes; small-diameter venules were sites of maximal adhesion and frequent blockage. Both FWV and ATSR peptides markedly decreased adhesion, and no vessel blockage was observed with either of the peptides, resulting in improved hemodynamics. ATSR also inhibited adhesion in unactivated microvasculature. Although infused fluoresceinated ATSR colocalized with vascular endothelium, pretreatment with function-blocking antibody to αVβ3-integrin markedly inhibited this interaction. Our data strengthen the thesis that ICAM-4 on sickle erythrocytes binds endothelium via αVβ3 and that this interaction contributes to vaso-occlusion. Thus peptides or small molecule mimetics of ICAM-4 may have therapeutic potential.

sickle cell disease; intercellular adhesion molecule-4; αVβ3-integrin; peripheral resistance unit; endothelium; erythrocytes

NORMAL CIRCULATING ERYTHROCYTES have little or no interactions with vascular endothelial cells. In marked contrast, convincing evidence is accumulating that, in sickle cell disease, continued presence of adhesion molecules on the surface of circulating sickle erythrocytes contributes to increased interactions between sickle erythrocytes and vascular endothelial cells leading to vaso-occlusion. A number of different protein associations have been defined, including 1) interactions of sickle reticulocyte α4β1 with VCAM-1 on cytokine-stimulated endothelial cells (30), 2) interactions between reticulocyte CD36 and endothelial cell vitronectin receptors via thrombospondin (9, 19, 29), and 3) von Willebrand factor (vWF) bridging of sulfated glycolipids exposed on sickle erythrocytes and αVβ3-integrin expressed on endothelial cells (4, 13, 19). There is also evidence linking red cell surface exposure of phosphatidylserine with sickle erythrocyte-endothelial adhesion (25). In addition, another category of erythrocyte adhesion receptors consists of molecules that require activation by signal transduction. Among these molecules are integrin-associated protein (CD47), intercellular adhesion molecule-4/LW (ICAM-4/LW), and basal cell adhesion molecule/Lutheran (B-CAM/Lu) expressed on both sickle reticulocytes and mature sickle erythrocytes (7, 14, 35). Activation of integrin-associated protein on sickle erythrocytes can induce signal transduction to activate yet unidentified cell receptors for thrombospondin (6). On the other hand, activation of B-CAM/Lu results in increased adhesion of sickle erythrocytes to laminin, an extracellular matrix protein (14).

The present study explores the role of ICAM-4 in sickle erythrocyte-endothelial interactions in the microcirculation contributing to vaso-occlusion. Erythrocyte-specific ICAM-4 is a member of the immunoglobulin superfamily that binds αV-integrin ligands (23). We and others have provided prior evidence that ICAM-4 may contribute to sickle erythrocyte-endothelial interactions via αV-integrin attachment. Although the endothelial cell receptor for ICAM-4 has not been definitively characterized, several investigations suggest that it may be αVβ3. Antibodies to αVβ3-integrin inhibit human sickle erythrocyte adhesion to platelet-activating factor (PAF)-treated rat endothelium under shear conditions and improve microvascular dynamics, suggesting that αVβ3 may be an endothelial cell integrin functioning in vaso-occlusion (19, 20). More recent in vitro studies revealed that epinephrine can act via a PKA-dependent pathway to activate ICAM-4-mediated sickle erythrocyte adhesion to endothelial cell αVβ3 (35). Interestingly, these latter studies support the novel concept that inside-out signaling mechanisms may activate red cell adhesion molecules in sickle cell disease.

In prior investigations, we identified a patch or “footprint” of amino acid residues on ICAM-4 that mediate adhesion to αV-integrins by performing targeted mutagenesis of surface-exposed residues, using a molecular model of ICAM-4 derived from the crystal structure of closely related ICAM-2 (23). The model of ICAM-4 presents the extracellular region as two...
Ig-like domains (1, 3): domain 1-situated NH2-terminal of membrane proximal domain 2. We found two neighboring sets of domain 1 residues, J) F18, W19, V20, and 2) R92, A94, T95, S96, R97, which mediate adhesion to αV.

To explore the contribution of ICAM-4 to vascular pathology in sickle cell disease, we tested the effects of peptides V(16)PFVVRMS (FWV) and T(91)RWTASRI (ATSR), composed of sequences within the ICAM-4 αV-binding region that are capable of inhibiting ICAM-4/αV integrin adhesion. Isolated rat mesoecum, a well-established ex vivo vasculature model that enables intravital microscopic observation and quantitation of sickle erythrocyte adhesion (15, 16, 19) was perfused with PAF, which is well known to induce endothelial oxidant generation (17, 28) and cause endothelial activation (22), both of which characterize sickle cell disease (10). PAF is elevated twofold in patients with sickle cell disease (24), and our group (19) showed that it enhances sickle cell adhesion in the ex vivo microcirculatory preparation. In PAF-perfused ex vivo mesoecum, treatment with peptides FWV and ATSR distinctly decreased sickle erythrocyte adhesion in venules of all diameters and vaso-occlusion in the microcirculation. In marked contrast, control peptide A(T76)WSSLAH(C) (AWSS) had minimal effect. Our data support the thesis that NH2-terminal domain 1 of ICAM-4 on circulating sickle erythrocytes binds endothelial cells via αβ3 and that this interaction significantly contributes to vaso-occlusion in sickle cell disease. Because platelets also express αβ3, at this time we cannot completely rule out the possibility that the observed sickle erythrocyte adhesive interactions with vascular endothelium may be mediated in small part by platelet-bridging mechanisms. Nonetheless, our findings suggest that peptides or small-molecule mimetics of ICAM-4 may have potential as therapeutic agents.

**METHODS**

**Peptide synthesis.** Synthetic peptides FWV, ATSR, and AWSS, comprising amino acids on the A, G, and F strands of ICAM-4 domain 1, respectively, were prepared. Peptides were synthesized on CLEAR resin (Peptides International, Louisville, KY) with the use of a standard 9-fluorenylmethoxycarbonyl protocol on a Pioneer peptide synthesizer (Applied Biosystems, Framingham, MA). Fluorophore-labeled peptides were prepared by attaching 5-carboxyfluorescein to the NH2 terminus of the resin-bound peptides. The peptides were cleaved from the resin, and side-chain protecting groups were removed simultaneously with Reagent B (TFA-phenol-water-TIPS: 88:15:2). The peptides were then purified to homogeneity by reverse-phase HPLC (Agilent Technologies, Palo Alto, CA) and analyzed with MALDI TOF mass spectrometer (Voyager DE, ABI, Foster City, CA). All peptides gave satisfactory mass spectrometry analysis.

**Preparation of sickle erythrocytes.** Heparinized blood was obtained with informed consent from crisis-free adult patients with sickle cell anemia (n = 8) who had not been transfused for at least 4 mo and who were not receiving hydroxyurea. The blood samples were drawn under a protocol approved by the Institutional Review Board (Albert Einstein College of Medicine, Bronx). After removal of the buffy coat, red blood cells were washed three times in normal saline, once in bicarbonate Ringer-albumin solution [118 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l CaCl2, 0.64 mmol/l MgCl2, 27 mmol/l NaHCO3, 0.5% BSA, equilibrated with 95% O2-5% CO2 (pH 7.4), osmolality adjusted to 295 mosmol/kgH2O] and resuspended in Ringer-albumin solution. In each case, the hematocrit was adjusted to 30% for perfusion studies.

**Preparation and perfusion of rat mesoecum vasculature.** Perfusion studies were performed in the isolated, acutely denervated, and artificially perfused rat mesoecum vasculature (n = 29) according to the method of Baaz et al. (2) as modified by Kaul et al. (16) for the infusion of erythrocytes. All experimental protocols were approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine. Briefly, perfusion pressure in the mesoecum was maintained at 60 mmHg, and venous outflow pressure was kept at 3.8 mmHg. During perfusion with Ringer-albumin solution containing 3% BSA, a 0.2 ml bolus of a sickle erythrocyte suspension (hematocrit, 30%) was infused via the arterial injection port over ~5 s. Peripheral resistance units (PRU) were determined as described previously (8) (expressed in mmHg·ml−1·min−1·g). PRU = ΔP/Q, where ΔP is the arteriovenous pressure difference and Q is the rate of venous outflow (ml/min) per gram of tissue weight. Pressure flow recovery time (Tpf), defined as the time (in seconds) required for the arterial pressure and the venous outflow to return to their baseline levels, was determined after the red cell infusion.

**Intravital microscopy and adhesion quantification.** Direct intravital microscopy and video recording of the microcirculatory events were carried out with a Nikon microscope (model E400; Nikon, Melville, NY) equipped with Dage-MIT-CCD television camera (model CCD-300T-RC; Dage-MIT, Michigan City, IN) and a Sony U-matic video recorder (model VOS800; Sony, Teaneck, NJ). The number of adherent sickle erythrocytes per 100 μm2 was determined from the counts of individual adherent cells and the surface area (μm2) of the inner wall of the vessel segment as described previously (15). Adhesion data for each experimental group were pooled for statistical comparisons.

**Experimental protocols with ICAM-4 peptides.** PAF supplied in chloroform solution (Sigma, St. Louis, MO) was first diluted in DMSO (Sigma), followed by serial dilutions in Ringer-albumin. The ex vivo mesoecum preparation was pretreated with 40 ml of Ringer-albumin containing PAF (200 pg/ml) for 10 min. The treatment with PAF was followed by infusion with 9-amino acid peptides FWV or ATSR (each 250 μmol/l) corresponding to ICAM-4 linear sequences that include amino acids critical for αV-binding sites (23). After 30-min incubation at room temperature, perfusion with 37°C Ringer-albumin was commenced immediately, and a bolus of washed oxygenated sickle erythrocytes was infused. Control preparations were similarly treated with PAF and AWSS (250 μmol/l). Erythrocytes from six individuals with sickle cell anemia were used in these experiments. In separate experiments using red blood cells from two additional patients with sickle cell anemia, we examined the adhesion of sickle erythrocytes in ex vivo preparations incubated 30 min with the peptide ATSR without prior PAF treatment.

**Immunofluorescence.** Fluoresceinated derivatives of ATSR and AWSS peptides were synthesized and infused employing the protocol described above. After a brief perfusion with Ringer-albumin (5 ml), the mesoecum tissue was frozen in tissue freezing medium (Miles Laboratories, Elkhart, IN) at −80°C. In other experiments, before infusion of fluoresceinated ATSR, the ex vivo mesoecum preparation was incubated for 30 min with MAb 7E3 (courtesy Dr. Barry S. Coller, Rockefeller University, New York, NY), which recognizes αβ3-integrin and inhibits sickle erythrocyte adhesion in this preparation or an irrelevant antibody OC125 that has no effect on sickle erythrocyte adhesion (19).

**Cryostat sections (7 μm thick) of freshly frozen mesoeicum tissue were postfixed in acetone. Sections were treated for 60 min at room temperature with primary rabbit anti-human vWF polyclonal antibody (Dakopatts, Glostrup, Denmark), a marker for endothelium, diluted 1:100 in PBS containing 5% BSA (Sigma). This was followed by a 60-min incubation in secondary antibody, tetramethidamine isothiocyanate-conjugated affinity-isolated swine anti-rabbit IgG (Dakopatts) at 1:60. After three washes in PBS, the sections were mounted with anti-fade medium (ProLong antifade kit; Molecular Probes, Eugene, OR) and visualized with a Nikon microscope (model 50i) equipped
RESULTS

Effect of αV-binding ICAM-4 peptides on the hemodynamic behavior of sickle erythrocytes in PAF-treated microvasculature. To explore the effect of ICAM-4 peptides on the vasocoercial potential of sickle erythrocytes, we tested sickle erythrocytes from six patients, utilizing the ex vivo mesocecum vasculature. In these experiments, we measured the PRU in the whole vasculature, while microcirculatory observations were carried out in the transilluminated mesocecum microvasculature.

We evaluated the hemodynamic behavior of sickle erythrocytes in preparations wherein PAF perfusion was followed by ICAM-4 peptide FWV or ATSR infusion. In each case, ΔPRU was expressed as the percent increase in PRU following the infusion of erythrocytes compared with PRU values measured after infusion of Ringer solution (Table 1). It should be noted that PAF infusion caused a modest increase in PRU compared with preparations perfused with Ringer-albumin alone in the absence of sickle red cells (4.3 ± 0.7 and 6.2 ± 1.2 mmHg·ml⁻¹·min⁻¹·g⁻¹ for Ringer vs. PAF-Ringer; \( P < 0.02 \)) (Table 1), which is in agreement with our previous studies (17, 19). It is likely that this is the result of PAF-induced increase in vascular resistance as described previously (19).

Infusion of sickle erythrocytes in PAF-treated preparations (group 2) resulted in a marked increase in ΔPRU values compared with the values observed in the untreated preparations (group 1), i.e., from 22.3 ± 3.6 to 34.1 ± 3.7 (\( P < 0.001 \)). The PAF-induced increase in PRU for sickle erythrocytes is in agreement with our previous measurements (17, 19). To determine the effect of blocking the interaction of ICAM-4 with αV, PAF treatment was followed by αV binding peptides. Incubation with peptides FWV (group 3) and ATSR (group 4) resulted in ΔPRU values of 19.4 ± 4.9 and 17.9 ± 5.9, respectively. These values were strikingly different from those measured for preparations treated with PAF alone (34.1 ± 3.7; \( P < 0.01 \)) and very similar to control group results (22.3 ± 3.6; not significant). In marked contrast, treatment with AWSS peptide (group 5), which is not a component of the αV binding footprint of ICAM-4, resulted in a pronounced increase in ΔPRU (32.5 ± 3.3) similar to that measured for preparations treated with PAF alone (34.1 ± 3.7).

The Tpf results were in agreement with the PRU data (Table 1). Infusion of sickle erythrocytes in PAF-treated preparations caused maximal increase in the Tpf values compared with the untreated preparations infused with sickle erythrocytes (Tpf increased from 38.0 ± 2.8 to 71.7 ± 14.4 s; \( P < 0.001 \)). The increase in Tpf was significantly lower in preparations pre-treated with either FWV (45.8 ± 10.7 vs. 71.7 ± 14.4 s; \( P < 0.001 \) or ATSR peptide (37.6 ± 9.9 vs. 71.7 ± 14.4 s; \( P < 0.005 \)). Treatment with control peptide AWSS resulted in Tpf values that were not different from the values for preparations treated with PAF alone (67.0 ± 5.7 vs. 71.7 ± 14.4 s; not significant). This showed again that, in contrast to the active peptides, AWSS peptide has no effect on the hemodynamic behavior of PAF-treated preparations.

Effect of αV-binding ICAM-4 peptides on sickle erythrocyte adhesion in PAF-treated microvasculature. Treatment with PAF caused prominent adhesion of sickle erythrocytes to endothelial cells (Fig. 1, A–D). As shown in Fig. 2A, an inverse correlation was noted between the number of adherent sickle erythrocytes and the vessel diameter (\( r = -0.81, P < 0.001 \), using the equation \( y = ax^{-b} \)). Furthermore, we noted frequent blockage of small-diameter postcapillary venules (Fig. 1D), as described previously (21).

Table 1. Effect of ICAM-4 peptides on the hemodynamic behavior of sickle erythrocytes in the ex vivo mesocecum vasculature

<table>
<thead>
<tr>
<th>Infusion</th>
<th>PRU, mmHg·ml⁻¹·min⁻¹·g⁻¹</th>
<th>Ringer</th>
<th>Erythrocytes</th>
<th>ΔPRU, %</th>
<th>Tpf, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sickle erythrocytes</td>
<td>5</td>
<td>4.3±0.7</td>
<td>5.3±0.8</td>
<td>22.3±3.6</td>
<td>38.0±2.8</td>
</tr>
<tr>
<td>2. PAF/Sickle erythrocytes</td>
<td>6</td>
<td>6.2±1.2</td>
<td>8.3±1.4</td>
<td>34.1±3.7</td>
<td>71.7±14.4</td>
</tr>
<tr>
<td>3. PAF/FWV/Sickle erythrocytes</td>
<td>6</td>
<td>6.0±1.2</td>
<td>7.2±1.5</td>
<td>19.4±4.9a</td>
<td>45.8±10.7a</td>
</tr>
<tr>
<td>4. PAF/ATSR/Sickle erythrocytes</td>
<td>5</td>
<td>5.8±2.2</td>
<td>7.0±3.1</td>
<td>17.9±5.9a</td>
<td>37.6±9.9a</td>
</tr>
<tr>
<td>5. PAF/AWSS/Sickle erythrocytes</td>
<td>5</td>
<td>6.3±1.0</td>
<td>8.3±1.2</td>
<td>32.5±3.4a</td>
<td>67.0±5.7a</td>
</tr>
</tbody>
</table>

Values are means ± SD. In these experiments, sickle erythrocytes were infused into untreated preparations (1), preparations were pretreated with PAF (200 pg/ml; 2), and preparations were pretreated with PAF followed by incubation with a given peptide (250 μmol each; 3–5). Sickle erythrocytes from 6 SS patients were tested: erythrocytes from 4 patients were tested in each infusion group. Because of insufficient blood sample erythrocytes from 1 patient were tested in groups 1, 2, 3, and 5, and erythrocytes from 1 patient were tested in groups 2, 3, and 4. No collected data points were excluded from our analysis. Statistical analysis carried out for 4 patients that were included in all experimental groups revealed similar trend of \( P \) values for infused peptide groups (groups 3–5) as compared with PAF group (group 2). ΔPRU, peripheral resistance units (PRU) for erythrocytes/PRU Ringer: Tpf, pressure flow recovery time to the baseline level. \( *P < 0.001 \) vs. sickle erythrocytes; \( \#P < 0.01 \) and \( \#P < 0.001 \) vs. respective values for PAF/sickle erythrocytes; \( \&P < 0.012 \) and \( \&P < 0.01 \) vs. respective values for PAF/sickle erythrocytes; \( @P < 0.005 \) vs. respective values, sickle erythrocytes, PAF/FWV/sickle erythrocyte and PAF/ATSR/sickle erythrocytes.
In marked contrast, when PAF perfusion was followed by infusion of peptide FWV or ATSR, adhesion of sickle erythrocytes was substantially inhibited in venules of all diameters (Fig. 2, B and C). In either case, a plot of untransformed data showed weak or no correlation of adhesion with venular diameter (FWV: \( r = -0.16, P < 0.172 \); ATSR: \( r = -0.25, P < 0.02 \)) (Fig. 2, B and C). Importantly, no postcapillary blockage was observed in either FWV- or ATSR-treated preparations (Fig. 1). In contrast to the findings with these two active peptides, adhesion of sickle erythrocytes in preparations treated with control peptide AWSS was essentially similar to the preparations treated with PAF alone (Fig. 1, E–H, and Fig. 2D).

Analysis of the sickle erythrocyte adhesion data shown in Fig. 2, B and C, revealed that the ATSR peptide inhibits sickle erythrocyte adhesion more effectively than FWV, as confirmed by a lower \( y \)-intercept value of the regression line of the transformed data (\( P < 0.001 \)). Further detailed analysis using logarithmic transformation of data confirmed the effectiveness of ATSR peptide in inhibiting sickle cell adhesion (Fig. 3A). The substantial inhibition of adhesion by ATSR compared with PAF alone-treated preparations is evidenced by a significantly
lower y intercept ($P < 0.001$), reflecting reduced numbers of adherent cells, and the markedly reduced slope of the regression lines ($P < 0.001$), demonstrating that this reduced adhesion is a feature of venules of all diameters. In contrast, the overall adhesion of sickle erythrocytes in preparations treated with control peptide AWSS was essentially similar to the preparations treated with PAF alone (Fig. 3B). Although a comparison of regression lines of the transformed data showed a marginally lower y intercept, there were no differences in the slopes of the regression lines.

Effect of ATSR peptide in preparations not activated with PAF. To determine the effect of ATSR on preparations that had not undergone PAF activation, we infused sickle erythrocytes from two additional patients into mesocecum preparations pretreated with only ATSR. We observed that APRU and Tpf values were not significantly different from those obtained for untreated preparations infused with sickle erythrocytes (data not shown). However, video analysis of intravital observations did show reduced adhesion in preparations treated with ATSR compared with those treated with Ringer solution alone (Fig. 4). Importantly, the decreased adhesion in ATSR-treated preparations showed no correlation with the vessel diameter ($r = 0.13, P = 0.36$).

Endothelial colocalization of αV-binding ATSR peptide and vWF. To confirm that the infused ATSR peptide was targeted to vascular endothelium, a fluorescent derivative of ATSR was infused in PAF-treated preparations. The ex vivo mesocecum vasculature was treated sequentially with PAF and then with the fluorescent peptide. Cryostat sections were treated with an anti-vWF antibody and a rhodamine-conjugated secondary antibody for identification of vascular endothelium. In sections from mesocecal preparations infused with the fluorescent peptide ATSR, we observed a distinct colocalization of ATSR with vWF in the vascular endothelium (Fig. 5, A–C). In marked contrast, preparations pretreated with fluorescent AWSS peptide showed no evidence of AWSS colocalization with vWF in the blood vessels (data not shown). When preparations were pretreated with MAb 7E3, which reacts with αVB3, infusion of fluorescent ATSR resulted in a striking decrease in colocalization with vWF (Fig. 5, G–I). However, in the presence of control antibody OC125, ATSR showed distinct colocalization with vWF (Fig. 5, D–F). Collectively, these observations demonstrate a specific interaction of ATSR peptide with vascular endothelial αVB3 receptors.

DISCUSSION

Our present data show that peptides based on αV-binding domains of ICAM-4 on sickle erythrocytes bind to endothelial cells via αVB3 and that this interaction can significantly contribute to vaso-occlusion in sickle cell disease. We showed marked improvements in the hemodynamic behavior of sickle erythrocytes in the ex vivo mesocecum preparations by infusion of synthetic mimetic peptides capable of inhibiting ICAM-4/αV-integrin binding. In preparations in which PAF treatment was followed by incubation with αV-binding peptide FWV or ATSR, infusion of sickle erythrocytes resulted in significant decreases in peripheral vascular resistance compared with preparations treated with PAF alone. In marked contrast, treatment with a control peptide had no effect. These observed changes in PRU were substantiated by the measured Tpf in the presence and absence of the specific peptides.

Also striking were our data obtained by direct microscopic observations and video analysis quantifying the effects of specific peptides on sickle erythrocyte adhesion. In preparations treated with PAF alone, adhesion of sickle erythrocytes showed a strong inverse correlation with venular diameter, which is in agreement with published observations from our
Adhesion was maximal in small-diameter venules, frequently resulting in vessel blockage. In preparations pretreated with PAF and the inhibitory peptides FWV and ATSR, sickle cell adhesion was substantially decreased in venules of all diameters. Importantly, we also observed that ATSR effectively inhibited sickle erythrocyte adhesion in ex vivo preparations that were not subjected to activation by PAF. Despite the fact that baseline sickle erythrocyte adhesion was reduced in the absence of PAF compared with that seen after activation by PAF, the ATSR peptide effectively abolished sickle erythrocyte adhesion. This finding lends strong support for a role of ICAM-4 in mediating sickle red cell adhesion.

We cannot, at this time, completely exclude the possibility that our preparations may contain platelets or platelet fragments. Hence, future investigations will be necessary to delineate whether a small portion of the observed ICAM-4-mediated sickle erythrocyte adhesive interactions with the vessel wall may be occurring via a bridging mechanism involving platelets, for example, via erythrocyte ICAM-4 adhesion to αVβ3 in platelets adherent to the vessel wall.

Fig. 4. Effect of peptide ATSR on sickle erythrocyte (SS RBC) adhesion in ex vivo mesocecum vasculature not pretreated with PAF. A: regression plot for the number of adhered sickle erythrocytes (SS RBC) in PAF-treated preparations and in preparations treated with PAF and peptide ATSR. Note the marked differences in both intercepts and slopes of the regression lines, demonstrating the pronounced inhibitory effect of peptide ATSR on sickle erythrocyte adhesion in venules of all diameters. B: similar comparison of regression lines revealed a minimal inhibitory effect of control peptide AWSS on sickle erythrocyte (SS RBC) adhesion compared with PAF-treated preparations.
Our present studies showed a more profound ATSR and FWV peptide-induced inhibition of adhesion than our group observed earlier (23). We attribute this to significant differences in methodology between the adhesion assays employed in the two studies. The earlier report utilized a static adhesion assay measuring HT 1080 cells binding to immobilized ICAM-4 in the presence and absence of blocking peptides. In contrast, the present assay was performed under flow that might remove more cells than static conditions. Additionally, the conformation of ICAM-4 in sickle cell membranes may be different than it is when immobilized as a monolayer, resulting in different steric relationships with its integrin-binding partner. Furthermore, presentation of the integrin-binding partner on HT 1080 cells may be different than it is on endothelial cells, resulting in variations in accessibility to blocking peptide. For these reasons, it is not surprising that the degree of blocking observed with the two different assays is not identical.

Important corroborating evidence for the vascular site of action of these peptides was provided by immunofluorescence studies wherein fluorescent ATSR peptide was infused into PAF-treated preparations. These experiments clearly demonstrated that binding ATSR peptide colocalized with vWF, a marker of endothelial cells. vWF-specific fluorescence was also evident in the subendothelial component of the vessel wall (see Fig. 5C). However, vWF colocalization with ATSR was specific to endothelium (the site of vWF production and its surface expression), where ATSR is likely to interact with the expressed αVβ3. Moreover, when mesocecal preparations were treated with function blocking αVβ3 antibody 7E3, ATSR peptide failed to colocalize with vWF. It should be noted that antibody 7E3 also recognizes GpIIbIIIa; however,
we do not believe that binding to GpIIbIIIa is playing a role in the present observations since our group (19) earlier reported that another MAb specific for GpIIbIIIa results in no significant differences in either PRU values or adhesion of SS erythrocytes in mesocellular preparations compared with control antibody or preparations treated with PAF alone. Hence, our present data strongly suggest that ATSR is localizing to microvascular endothelium via binding to αVβ3 integrin. As discussed, future studies will determine whether there is any mechanistic role for platelet bridging in this process.

In both human with sickle cell disease and transgenic sickle mice, chronic inflammatory activation of endothelium results in upregulation of endothelial adhesion molecules (i.e., VCAM-1, ICAM-1, E-selectin, P-selectin, and αVβ3-integrin) (5,10,26,32). However, the mechanistic aspects of red blood cell adhesion in transgenic sickle mice are not well understood, and the substances that stimulate or inhibit red cell adhesion in transgenic mice are yet to be characterized. In contrast, the ex vivo model used in the present investigations, similar to cultured human endothelial cells, allows testing known stimulating and inhibitory agents on adhesion of human SS red blood cells (4, 18, 19). Importantly, the ex vivo model allows distinction of microvascular sites and characteristics of adhesion and vaso-occlusion under conditions of shear flow. Thus the use of the ex vivo preparation is relevant to identify the ICAM-4 domains involved in human SS red blood cell adhesion in the microcirculation. It would be important in future studies to validate these findings in relevant sickle mouse models, as well as establish the contribution of plasma factors and other blood cells to ICAM-4-mediated sickle vaso-occlusion.

We and others have shown that ICAM-4 is unique among the ICAM family in that it interacts with multiple integrins, including αLβ2 (LFA-1) and αMβ2 (Mac-1) on leukocytes (12), the fibrinogen receptor αIIbβ3 (GpIIb/IIIa) on platelets (11), as well as αβ1- and αV-integrins (27,35). Thus ICAM-4 has multiple domains that bind diverse integrins, some of which may have important roles in cell-cell interactions in sickle cell disease. Hence, the possibility exists that ICAM-4 may be involved in various dynamic cell-cell interactions during vaso-occlusion, including sickle red cells with endothelial cells, neutrophils, and platelets. The spatial relationship of integrin binding sites on the surface of ICAM-4 is emerging. Interestingly, the area on ICAM-4 important for its interaction with αV-integrins is adjacent to, but distinct from, the binding sites previously identified for the ICAM-4-binding partners αLβ2 and αMβ2.

Although future work will be required to explore whether ICAM-4 binding to platelet and neutrophil receptors may also contribute to vaso-occlusion (31,33,34), the present studies appear to show that αV-binding peptides of ICAM-4 largely target microvascular endothelium and inhibit sickle erythrocyte-endothelium interactions and vaso-occlusion. Our approach indicates that unwanted interactions associated with pathology can be ameliorated without compromising other functions. Moreover, these findings suggest that peptides or small-molecule mimetics of ICAM-4 may have potential as therapeutic agents for the treatment for sickle cell crisis.

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