Lactadherin mediates sickle cell adhesion to vascular endothelial cells in flowing blood

Increased exposure of sickle red blood cells to phosphatidylserine promotes its adhesion to the endothelium. A monoclonal antibody to lactadherin, a phosphatidylserine binding protein, inhibits sickle cell adhesion to histamine-stimulated endothelial cells in flowing blood. Added lactadherin enhances the adhesion via the integrin αVβ3. These results indicate that lactadherin can mediate phosphatidylserine-expressing sickle cell adhesion to the endothelium.

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In sickle cell anemia, there is a strong correlation between the red cell adhesion to endothelium and clinical severity.[1] Increased exposure of sickle red blood cells to phosphatidylserine is one the factors that promote adhesion[2]. Lactadherin (also known as milk fat globule-EGF factor 8), is a phosphatidylserine-binding glycoprotein containing an EGF-like domain at the amino terminus with the RGD sequence and two C-domains at the carboxy terminus. It promotes engulfment of phosphatidylserine-containing apoptotic cells[3] and sickle red blood cells[4] by macrophages. We have examined its role in sickle cell adhesion to the endothelium.

Bovine Lactadherin was isolated as described before.[4,5] The monoclonal antibody L688 (IgAκ) was derived from a mouse immunized with bovine lactadherin.[6] The splenocytes were harvested and fused to non-secreting myeloma cell line NS-1. The resulting hybrids were screened for antibody production against human recombinant lactadherin by ELISA. One hybrid that showed significant binding was expanded, retested, and cloned by limiting dilution. The antibody was purified from the tissue culture supernatant by ammonium sulfate precipitation and gel filtration on Sephacryl S 200 (Amersham-Pharmacia, Piscataway, NJ, USA).

After receiving their written informed consent, blood was collected from volunteers or patients with sickle cell anemia (homozygous SS). The study was approved by the committee for the protection of human subjects at the Baylor College of Medicine. None of the patients were clinically in a pain crisis. Washed red blood cells were incubated with N-ethylmaleimide (10 mM) and ionophore A23187 (4 μM) to induce transbilayer movement of phosphatidylserine as described by Kuypers *et al.*[2] A parallel-plate flow chamber was assembled with the histamine-treated endothelial cells forming the bottom of the flow chamber and mounted onto an inverted-stage microscope equipped with a high-speed digital camera as previously described.[7] Washed red cells, resuspended at 1% hematocrit in autologous plasma or in serum-free medium 199, containing 1% bovine serum albumin, were perfused through the chamber to generate a wall shear stress of 1.0 dyne/cm^2 for five minutes and the images of adherent red cells were acquired. These images were analyzed offline using MetaMorph software (Universal Images, West Chester, PA) in 15 random fields at 40× magnification. The adhesion is expressed as the number of red blood cells in a field.

All experimental values are represented as mean ± standard error. Analysis of the difference was performed using Student's t-test for paired and unpaired data. Values were regarded significant at $p<0.05$.

Sickle red blood cells, in autologous platelet-free plasma, adhered to histamine-treated human umbilical vein endothelial cells avidly at the venular shear stress of 1.0 dyne/cm^2 in a parallel-plate flow chamber as described.[1] L688 significantly inhibited the adhesion compared to an isotype matched control monoclonal antibody MOPC-320 (Figure 1A). In five separate experiments involving five patients, L688 inhibited the adhesion by 24-30% ($p<0.001$). These results show that lactadherin is one of the molecules that mediate adhesion of sickle red blood cells to the endothelium under flow in a whole blood milieu. In a separate set of experiments, we examined the adhesion in a serum-free tissue culture medium. Lactadherin stimulated sickle red blood cell adhesion to endothelial cells in a concentration-dependent manner

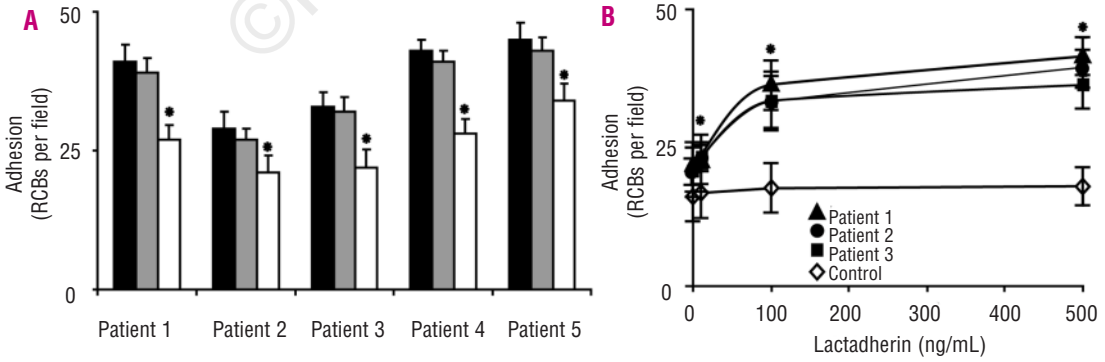

Figure 1. A. Inhibition of sickle cell adhesion to endothelium by antilactadherin antibody. Washed sickle red blood cells, resuspended in autologous plasma (1% hematocrit), were perfused over histamine-stimulated HUVEC monolayers at a shear stress of 1.0 dyne/cm^2 for 5 minutes in a parallel plate flow chamber. The adherent red blood cells were quantified and the adhesion is expressed as the number of red blood cells per high power field. Closed bar, buffer without any antibody; gray bar, control antibody MOPC-320 (20 μg/mL) and open bar, antilactadherin antibody L688 (20 μg/mL). The results are the means and standard errors of measurements in 15 random fields. *denotes a $p<0.001$ when compared to buffer. **B.** Washed sickle red blood cells, resuspended in serum-free medium 199, were perfused at a shear stress of 1 dyne/cm^2 over histamine-stimulated endothelial cell monolayer for 5 minutes. Adhesion was measured at various concentrations of lactadherin as described in methods. The results are expressed as means and standard errors for each patient. *denotes a $p<0.001$ when compared to buffer.

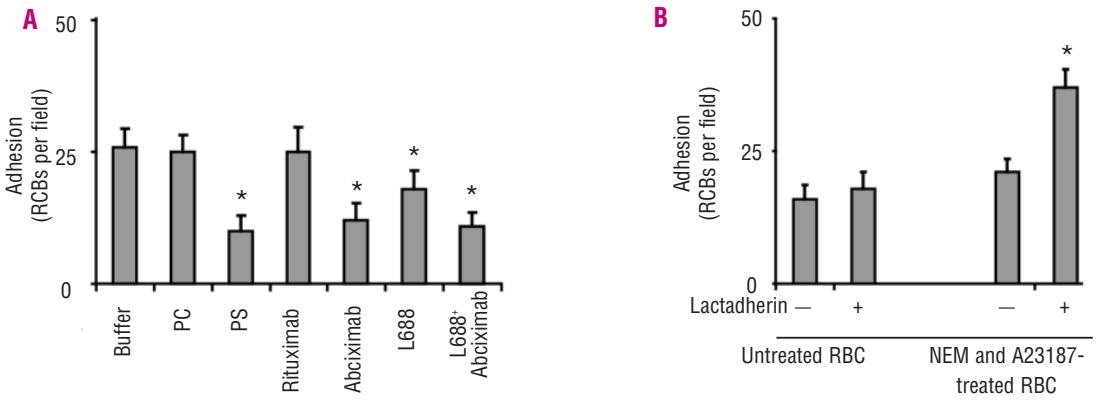

Figure 2. Panel A. Inhibition of sickle red blood cell adhesion to endothelial cell monolayers. The lactadherin-dependent adhesion of washed sickle red blood cells to stimulated endothelial cell monolayers was measured at a shear stress of 1 dyne/cm² in the presence of various agents (20 μg/mL). PC, phosphatidylcholine vesicles. PS, phosphatidyl-serine vesicles. *denotes a *p* value of <0.05 when compared to buffer. Panel B. Effect of lactadherin on the adhesion of phosphatidylserine-expressing normal red blood cells to endothelial cells. Washed normal red blood cells were treated with ionophore A23187 and N-ethylmaleimide and perfused at a shear stress of 1 dyne/cm² over histamine-stimulated endothelial cell monolayer for 5 minutes. Red blood cell adhesion was measured in the presence or absence of lactadherin (10 μg/mL). * denotes a *p* value of <0.05 when compared to buffer.

(Figure 1B).

Lactadherin-dependent adhesion of sickle red blood cells was inhibited by abciximab, an antibody fragment that targets the β subunit of integrin αVβ3 (Figure 2A). Furthermore, phosphatidylserine vesicles also inhibited sickle cell adhesion. These results show that lactadherin bridges the phosphatidylserine-expressing sickle red blood cells and endothelial cells by binding to phosphatidylserine on red blood cell surface and integrin αV,3 on the endothelial cell surface.

Lactadherin had no effect on the basal adhesion of normal red blood cells (Figure 2B). However, when normal red blood cells were pretreated with N-ethylmaleimide and calcium ionophore A23187, which induces transbilayer movement of phosphatidylserine from the inner to the outer leaflet of the plasma membrane bilayer,[8] the addition of lactadherin significantly increased adhesion (Figure 2B). The abnormal adhesion of sickle cells to vascular endothelium involves multiple ligands and receptors and correlates strongly with severity of vasoocclusive crisis.[1] Several studies have also shown that sickle cells express a significant quantity of phosphatidyl-serine on their outer surface.[2,9]

The data presented here show that lactadherin, which is present in plasma,[10] is a mediator of phosphatidyl-serine-expressing sickle cell adhesion to the endothelium under physiological venular shear stress via αVβ3. Lactadherin also promotes the phagocytosis of phosphatidylserine-expressing sickle red blood cells by macrophages.[4] We suggest that lactadherin-bound sickle red blood cells that escape ingestion by macrophages are more adhesive towards endothelium. In conclusion, lactadherin appears to be involved both in sickle cell clearance from the circulation and in adhesion to the endothelium.

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