Intercellular adhesion molecule-4 and CD36 are implicated in the abnormal adhesiveness of sickle cell SAD mouse erythrocytes to endothelium

Marie-Marcelle Trinh-Trang-Tan,[1,2,3] Camilo Vilela-Lamego,[2] Julien Picot,[1,2,3] Marie-Paule Wautier,[1,2,3] and Jean-Pierre Cartron[1,2,3]

[1]Inserm, U665, Paris; [2]Institut National de la Transfusion Sanguine, Paris, and [3]Université Paris7/Denis Diderot, Paris, France

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Supplementary Design and Methods

SAD mice

Animal experiments were approved by the Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture (Paris, France). Mice of both genders aged 2 to 6 months old were used.

Reagents

Cell culture media and reagents were from Gibco BRL. Except when otherwise mentioned, reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The osmolality of phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS) was adjusted to 320 mOsm/kg H_2O by addition of NaCl, to prevent hemolysis of mouse RBC. HBSS contained 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Peptides T(91)REATARI (T-8-I) and A(76)WNSLAHC (A-C-8, control) were synthesized, purified by reverse phase high performance liquid chromatography, and analyzed by matrix assisted laser desorption ionization – time of flight (MALDI TOF) mass spectrometry (Genosphere Biotechnologies, Paris, France). These peptides are the mouse counterparts of the peptides used by Kaul and co-workers to inhibit sickle cell-endothelial interactions and vaso-occlusion in rat mesocecum.[1]

Cyclic AMP concentrations were measured using a competitive enzyme immunoassay kit (Cayman Chemical, Ann Harbor, MI, USA). Mouse tumor necrosis factor-α was from BD Biosciences Pharmingen.

Glass microslides were purchased from Camlab (Cambridge, UK).

Antibodies

The expression of adhesion molecules on RBC and endothelial cell surfaces was evaluated by flow cytometry using the following antibodies purchased from BD Biosciences Pharmingen: Armenian hamster monoclonal antibodies against mouse CD29 (β1), CD61 (β3), and CD54 (ICAM-1) (respectively, clones HMβ1-1, 2C9.G2, and 3E2); rat monoclonal antibodies against mouse CD49d (α_4), CD51 (α_v), CD106 (VCAM-1), CD47, CD62E (E-selectin), CD62P (P-selectin), CD147 (respectively, clones R1-2, RMV-7, 429, miap301, 10E9.6, RB40.34, and RL73); and mouse monoclonal antibodies against mouse CD36 (clone JC63.1).

The rabbit polyclonal anti-mouse ICAM-4 antibody (Lp38267) was raised against a synthetic peptide (sequence SVTSAPFWVRL-NPELEAV) coupled to keyhole limpet hemocyanin (NeoMPS, Strasbourg, France). The peptide sequence corresponds to the mouse counterpart of one of the human ICAM-4 domains interacting with $\alpha_v\beta_3$ integrins.[2,3] The antibody specificity was characterized by western blotting using proteins from plasma membranes, obtained after hypotonic lysis, of collected RBC. The rabbit polyclonal anti-mouse Lu/BCAM (antibody 455) has been characterized elsewhere.[4] The rabbit polyclonal antibody anti-CD36 was purchased from Cayman Chemicals. Rabbit polyclonal antibody against Band3 was a gift from Dr L. Peters (The Jackson Laboratory, Bar Harbor, ME, USA).

Agarose-conjugated monoclonal anti-phosphothreonine (clone PTR–8) and anti-phosphotyrosine (p-Tyr-100) antibodies were purchased from Sigma (St. Louis, MO, USA) and Cell Signaling Technology (Danvers, MA, USA) respectively.

Cell culture

The bEnd.3 cell line was established from a polyoma-induced hemangioma in brain.[5] Cells were grown in Iscove's modified Dulbecco's medium (IMDM) containing 10% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin, and exhibit the typical cobblestone aspect at confluence. The bEnd.3 cells were used at passages 24 to 29.

Purification and labeling of red blood cells

Blood samples (50-150 μL), were collected by retro-orbital bleeding. Purified erythrocytes were recovered after centrifugation on Ficoll-Paque Plus (Amersham), elimination of white cells, and repeated washes of the pellets of RBC with HBSS. We verified that PKH67 labeling for 2 min, and PKH 26 labeling for 1 min did not alter the adhesion properties of RBC by comparing adhesion of labeled and unlabeled RBC on confluent endothelial cell monolayers in separate experiments (*data not shown*).

Flow cytometry analysis

Cells were incubated in PBS containing 0.2% bovine serum albumin and 5 μg/mL of each monoclonal antibody, for 60 min at 22°C. After extensive washes they were stained with phycoerythrin-conjugated goat anti-rat or hamster IgG (Fab'2). The binding of the monoclonal antibody was quantified on 0.5×10^6 cells by flow cytometry (FACSCalibur flow cytometer, Beckton-Dickinson). Thiazole

orange was used to estimate the percentage of reticulocytes among the total erythrocyte population. As mouse adhesion receptors were investigated here with the use of monoclonal antibodies raised in rat or Armenian hamster, it was not possible to quantify them in terms of copy number per cell. Their levels are, therefore, expressed as mean fluorescence intensity (MFI).

Red blood cell membrane preparation

The whole procedure was conducted at 4°C and used ice-cold solutions. Freshly collected RBC were washed three times with PBS and lysed with 40 volumes of hypotonic solution (5 mM Na_2PO_4 pH 8.5, 0.35 mM EDTA, 1 mM AEBSF as a protease inhibitor).[6] In immunoprecipitation studies, cocktails of serine/threonine or tyrosine phosphatase inhibitors were added according to the manufacturer's instructions (Sigma-Aldrich). Cell membranes were recovered after five washes with hypotonic solution.

Immunoblotting

Membrane proteins (10-20 μg) were separated in SDS-10% polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membrane (NEN). Blots were blocked with blocking buffer (Sigma) at 4°C overnight. They were then incubated for 60 min at room temperature in blocking buffer containing mICAM-4 antiserum (dilution 1:2000). After three washes in a first solution (0.1M Tris-HCl, pH 7.4; 0.15 M NaCl; 0.3 % Tween 20), and two washes in a second solution (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl), blots were incubated for 40 min in blocking buffer containing goat anti-rabbit IgG conjugated with horseradish peroxidase (Promega). Specific bands were revealed by enhanced chemiluminescence (ECL+, Amersham). N-glycanase F from Biolabs was used for protein deglycosylation before gel loading. The specificity of our anti-mICAM-4 antiserum was confirmed by using, as primary antibodies, either the antiserum saturated overnight by the immunizing peptide, or pre-immune serum (dilution 1:2000).

Adhesion assay under flow conditions

A suspension of bEnd.3 cells ($6x10^6$/mL IMDM with 10% FCS) was injected into gelatine (2%)-coated glass flat capillaries (microslides, Camlab, Cambridge, UK) and left to adhere for 2 h. Cells were then grown to confluence for 48 h with replacement of the capillary medium every 2 h.

The microslides were mounted on the stage of a video camera equipped stereomicroscope (Leica Microsystem). One end of the microslide was connected to a syringe pump (Harvard Apparatus, Holliston, MA, USA) to control the flow rate that determines shear stress levels. The other end was connected to an electronic valve allowing switching between the RBC suspension and a cell-free washing buffer (HBSS). The differently fluorescent experimental and control RBC were mixed in HBSS to reach a final hematocrit of 0.2% each, before injection into microslides. The RBC mixture was injected into the microslide at a flow rate that produced a wall shear stress of 0.02 Pa for 5 min. The RBC that did not adhere to the endothelial monolayer during the infusion period were removed by infusion of cell-free HBSS at the same shear stress for 5 min, and then stepwise at increasing shear stresses of 0.04, 0.1, 0.2, and 0.4 Pa, every 5 min. At the end of each wash period, pictures of nine consecutive fields of known surface area along the center line of the microslide were recorded under fluorescent filters adequate for discriminating experimental from control RBC (Optimas G.S., Silver Spring, MD, USA). RBC were counted using NIH image software.

Adhesion assays were conducted under basal conditions and under conditions in which RBC and/or endothelial cells were treated. RBC were not treated, treated with 50 nM of epinephrine for 2 min, or incubated with monoclonal antibodies against mouse CD36 (10 μg/mL) for 30 min. Endothelial cell monolayers were not treated, treated with synthetic peptides (250 μM), or treated with monoclonal antibodies against mouse α_v integrin (20 μg/mL) for 30 min. Endothelial monolayers were also activated with platelet activating factor (0.2 ng/mL) for 10 min.

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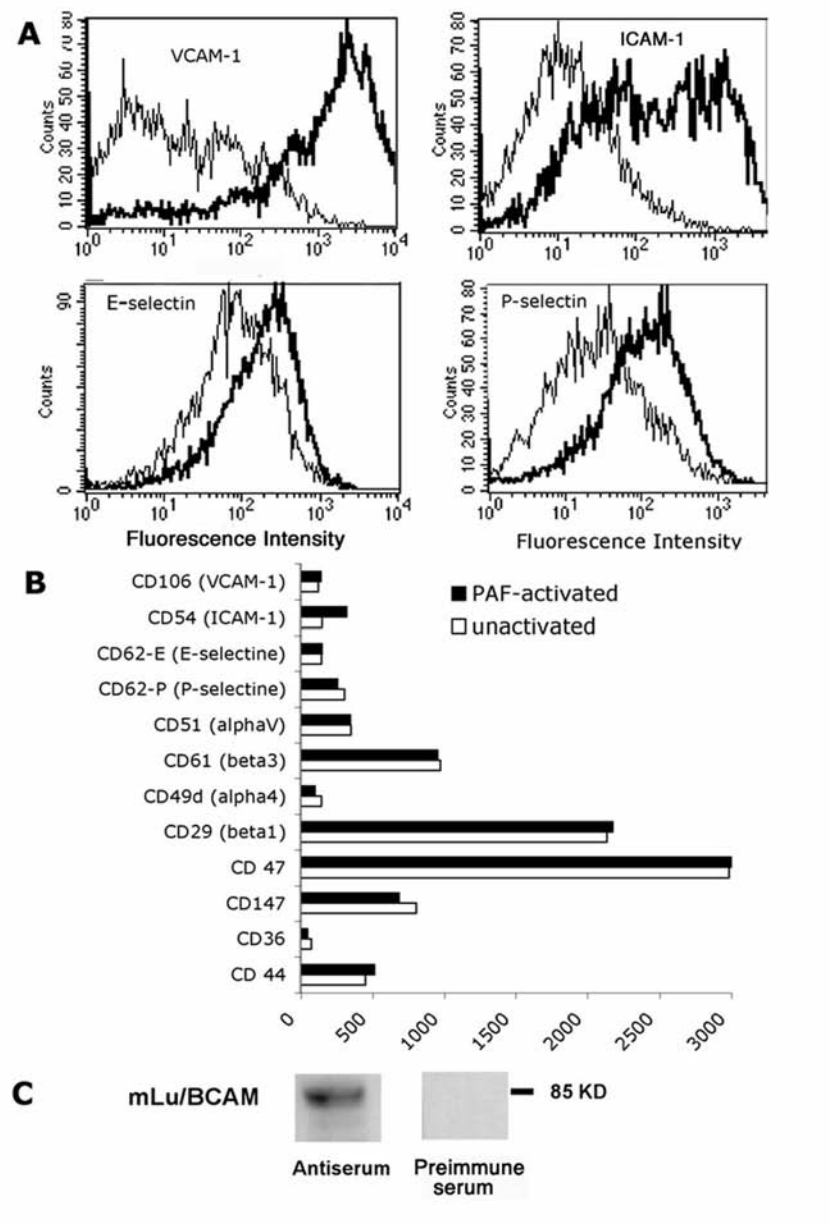

Online Supplementary Figure S1. Adhesion molecules on resting and activated bEnd.3 endothelial cells: (A) Flow cytometry analysis of the effect of murine TNFα (5 ng/mL): The levels of expression of ICAM-1, E-selectin, and P-selectin, are increased (heavy lines) following exposure to TNF-α for 4 h, and that of VCAM-1 after exposure for 16 h. (B) Flow cytometry analysis of the effect of PAF (0.2 ng/mL). (C) Immunoblotting detection of Lu/BCAM as a 85 KD glycoprotein. The absence of a band with the pre-immune serum indicates the specificity of the antibody.