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Activation of human neutrophil Mac-1 by anion substitution

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ABSTRACT

Substituting the medium chloride with glucuronate or glutamate causes a rapid, 10 to 30-fold, increase in the binding of the monoclonal antibody, CBRM1/5, which recognizes the high-affinity conformation of the Mac-1 integrin. This change is reflected in functional adhesion assays that show increased adhesion to ICAM-1 coated beads. Blocking antibodies indicate that the increased adhesion is almost entirely due to Mac-1. The inhibitor NPPB (100 μM) reduces Cl^- efflux into low Cl^- medium by 75%, and blocks increased CBRM1/5 binding after stimulation with fMLP or TNF-α, but has no effect on the anion substitution induced increase in CBRM1/5 binding or adhesion to immobilized ICAM-1. Thus, changes in *external* anion composition, not internal chloride or increases in Cl^- efflux, are responsible for Mac-1 activation. This effect is substantial. The percentage of Mac-1 in the high affinity state approaches 100% in glutamate and 50% in glucuronate, a far greater response than what is observed after stimulation with fMLP.

Introduction

A critical step in the process by which neutrophils become selectively localized at sites of inflammation involves firm adhesion of receptors on the neutrophil to ligands on endothelial cells lining post-capillary venules, enabling neutrophils to migrate toward the site of inflammation rather than being swept away in the blood. Firm adhesion is triggered by conformational changes in neutrophil integrins ("activation") that increase their affinity for binding to endothelial cell ligands such as intercellular adhesion molecule (ICAM-1) [1,2]. The importance of cations in integrin binding to ligands has long been recognized. Cations such as Mg^{2+} or Mn^{2+} can bind to the external portions of integrins [3–6], thereby causing conformational changes that are detected by binding of certain monoclonal antibodies, such as mAb24 [4,7] or CBRM1/5 [8,9], and are associated with increased affinity for ligands [6,10]. Crystal structures of integrins show several divalent cation binding sites, including the metal ion dependent adhesion site (MIDAS) in the I domain of the α subunits of many integrins, and the adjacent to MIDAS (ADMIDAS) site in the I-like domain of the β subunits [1].

While the importance of cations in regulating integrin conformation is widely recognized, the possible effects of anions on integrin conformation are relatively unexplored. The crystal structures of integrins in the ligand-bound forms show anionic amino acids, glutamate or aspartate, bound at the ligand binding site [9,11,12], yet the possibility that introduction of these amino acids into solution might affect integrin conformation has not been thoroughly tested. In the present report we demonstrate that inorganic anions in the cell environment can have a direct effect on integrin conformation and affinity. We use the activation-sensing monoclonal antibody CBRM1/5, which binds specifically to a portion of the I domain of the α_M chain [8,9], to detect effects of activators and changes in external anion composition on Mac-1 conformation. We also document corresponding changes in integrin affinity by measuring neutrophil adhesion to beads coated with the endothelial ligand, ICAM-1.

Materials and methods

Reagents

Polymorph Cell Separation Medium was obtained from Accurate Chemical and Scientific Company. Bovine serum albumin (BSA), D-glucuronic acid sodium salt, L-glutamic acid monosodium salt hydrate, NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid), and fMLP were obtained from Sigma (St. Louis, MO). Human rTNF-α and ICAM-1 were purchased from R and D Systems (Minneapolis, MN).

Antibodies

Monoclonal antibodies ICRF44 (anti-human CD11b, IgG1), which binds to the α_M subunit and blocks Mac-1 ligand binding, R-PE-labeled and unlabeled mAb38 (anti-CD11a, IgG2a), which binds to the α_L

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subunit and blocks LFA-1 ligand binding, R-PE-labeled and unlabeled IB4 (anti-CD18, IgG2a), which binds to the β_2 subunit and blocks CD18 ligand binding, R-PE-labeled IgG2a isotype control and FITC conjugated mAb 15.2 (anti-human ICAM-1, IgG1) were purchased from Ancell (Bayport, MN). KIM127, which maps to I-EGF domain 2 in the β2 leg, and AL57 Fab fragments, which report LFA-1 activation, were kindly provided by Dr. Minsoo Kim (University of Rochester, Rochester, NY).MAb 24 was a generous gift from Nancy Hogg (Cancer Research UK London Research Institute, London, UK). AL57 Fab fragments were labeled with AlexaFluor 488 antibody labeling kit (Invitrogen/Molecular Probes, Eugene, OR). FITC-labeled mAb CBRM1/5, which binds to the activated form of the I domain of human Mac-1 (CD11b/CD18) and FITC-labeled rat anti-mouse IgG were purchased from eBioscience (San Diego, CA). PE anti-human CD11b (Bear 1), PE-labeled IgG1 Isotype control, and FITC-labeled IgG1 isotype control were obtained from Beckman Coulter Immunotech (Miami, Fl). HUTS4 (IgG2b), which is specific to the active conformation of β_1 integrin, was purchased from Millipore (Temecula, CA). FITC-labeled MEM-101A (anti-human CD29, IgG1), which binds to the β_1 subunit, was purchased from Invitrogen Corporation (Carlsbad, CA). FITC-labeled anti-mouse IgG2b and FITC-labeled anti-mouse IgG1 were purchased from BioLegend (San Diego, CA). Quantum Simply Cellular Beads were purchased from Bangs Laboratories, Inc. (Fishers, IN).

Neutrophil isolation

Neutrophils were isolated from the whole blood using the Ficoll–Hypaque method [13]. After centrifugation the neutrophil-containing layer was collected, restored to normal osmolality by addition of an equal volume of balanced salt solution (BSS) (146 mM NaCl, 5 mM KCl, 5.5 mM D-glucose, 10 mM (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (HEPES)) that had been diluted 1:1 with distilled, deionized water, and then resuspended in 5 ml of BSS with 0.1% BSA, pH 7.4. The cells were washed three times with this buffer, centrifuging for 10 min at 950 rpm (105 g) in an Eppendorf microcentrifuge. The cells were then resuspended at 5×10^6 cells per ml in BSS + 0.1% BSA buffer containing 1 mM $CaSO_4$ and 1 mM $MgSO_4$.

Low chloride experiments with CBRM1/5 antibody and NPPB

Neutrophils at 5×10^6 cells per ml were centrifuged in an Eppendorf microcentrifuge at 1500 rpm (165 g) for 5 min at room temperature and were resuspended to the original cell concentration in either BSS + 0.1% BSA with 1 mM Ca^{2+} and Mg^{2+} (as above), or in low chloride (low Cl^-) buffer (150 mM sodium glucuronate or 150 mM sodium glutamate or 150 mM sodium gluconate, 5.5 mM D-glucose, 10 mM Hepes, 1 mM $CaSO_4$, and 1 mM $MgSO_4$, pH 7.4) with 0.1% BSA, or at intermediate concentrations of glutamate or glucuronate substituted for Cl^-. Aliquots (100 μl) were taken immediately upon resuspension and at designated time points and placed on ice to cool. To assay the expression of epitope CBRM1/5, the cell suspensions were incubated for 1 h at 4 °C with FITC-labeled CBRM1/5 mAb (15 μg/ml). To assay total CD11b expression, cells were incubated for 45 min at 4 °C with a saturating level (20 μl of the solution provided by the manufacturer) of PE anti-CD11b (Bear-1). Cells were washed three times in either low Cl^- buffer or BSS with 0.1% BSA at 4 °C and fixed in 1% paraformaldehyde. Prior to flow cytometry, fixed cell samples were centrifuged once and resuspended in BSS.

In experiments with NPPB, 5×10^6 cells per ml were pretreated with the inhibitor for 5 min at 21 °C in BSS + 0.02% BSA, followed by a 5 min centrifugation at 1500 rpm (165 g) at 21 °C. Neutrophils were resuspended to original volume in low Cl^- buffer or BSS with 0.02% BSA, with or without 100 μM NPPB. Cells were incubated for an additional 2 min at 37 °C or room temperature and then 100 μl aliquots were placed on ice to cool prior to antibody labeling. For micropipette studies, 4% fetal bovine serum (FBS) was substituted for 0.02% BSA and after labeling, cells were placed directly into the chamber on the microscope stage. In experiments with NPPB and stimulating agents, neutrophils were treated with 100 μM NPPB for 5 min followed by 15 min incubation at 37 °C with 10 nM fMLP or 20 ng/ml TNF-α.

Flow cytometry

Samples were analyzed in an Epics Elite (Coulter Instruments) flow cytometer or FACS Calibur (Becton Dickinson). Gates were set based on forward and side scatter to exclude red blood cells. 10,000 cells were analyzed for each sample. To correlate fluorescence intensity with the number of bound antibodies on cells or beads, the fluorescence signal was calibrated using Quantum Simply Cellular Beads (Bangs Laboratories, Inc., Fishers, IN) [13]. These beads provide a calibration of the Mean Fluorescent Intensity in terms of the number of antibodies bound to the surface. Thus, binding is expressed in terms of the antibody binding capacity, a quantitative measure of the number of antibody binding sites on the cell surface.

Chloride efflux in low Cl^- medium

Isolated neutrophils were washed twice in BSS, pH 7.4, with 0.1% BSA, at room temperature. The remaining red cells were lysed by resuspending the preparation in hypotonic buffer (14% v/v of Dulbecco's PBS without calcium or magnesium (Gibco) in distilled water) for 30 s, followed by the addition of 4× hypertonic PBS containing the appropriate amount of BSA to give a final isotonic solution with 0.1% BSA. Cells were then centrifuged, washed once in BSS containing 0.1% BSA, and resuspended to 2×10^7 cells per ml in the same buffer, except with the pH titrated to 7.4 at 37 °C, for incubation with 2.5 μCi/ml $^{36}Cl^-$ for 1.5–2 h at 37 °C. For each flux measurement, 0.9 ml of this suspension was washed twice in BSS containing 0.1% BSA at room temperature to eliminate excess isotope. Isotope-loaded cells were resuspended in 4.5 ml sodium glucuronate buffer (153 mM sodium glucuronate, 1.5 mM $CaSO_4$, 1 mM $MgSO_4$, 5.5 mM D-glucose, 10 mM HEPES, pH 7.4 at 21 °C) containing 0.1% BSA at 37 °C. If NPPB was present, only 0.01% BSA was used. At each predetermined time interval, 0.5 ml cell suspension was layered over 0.5 ml of a mixture of Silicone AR 200 fluid (Serva) and mineral oil (Sigma), with density 1.03 g/ml, in a 1.5 ml Eppendorf centrifuge tube. The sample was centrifuged immediately at 12,500 rpm (12,800 g) for 30 s in an Eppendorf microcentrifuge; sample time was recorded as the start of centrifugation. A 0.3 ml sample of the supernatant was taken, mixed with 0.4 ml SDS (0.1%), and added to 5 ml scintillation fluid to count for 10 min. Replicate 0.3 ml samples of the cell suspension were added directly to SDS and scintillation fluid to determine the total count per minute (cpm) in the suspension, y_{inf}. The rate constant for Cl^- efflux was calculated by fitting the cpm (y) at various times (t) to the equation $y = y_0 + (y_{inf} - y_0)\,(1 - \exp(-kt))$, where y_0 is the cpm at time zero. Values of y_0 and k were determined from a non-linear least squares fit using the program Origin (Microcal), with y_{inf} held constant at the measured value.

Coating beads for micropipette experiments

Recombinant soluble ICAM-1 (R and D Systems, Minneapolis, MN) was coupled covalently via tosyl linkage to paramagnetic M-450 Dynabeads (Dynal, Lake Success, NY) [6].

The density of ICAM-1 on ligand-coated beads was measured by flow cytometry. The beads were preincubated at 4 °C overnight with FITC-conjugated mAb 15.2 against human ICAM-1. The beads coated with ethanolamine were used to detect background fluorescence. To correlate fluorescence intensity with the number of bound antibodies on cells or beads, the fluorescence signal was calibrated using Quantum Simply Cellular Beads. Beads used in these experiments had an ICAM-1 surface density of 250 or 140 sites/μm^2.