Blood Cells, Molecules, and Diseases 46 (2011) 246–251

Contents lists available at ScienceDirect

Blood Cells, Molecules, and Diseases

journal homepage: www.elsevier.com/locate/ybcmd

Role of c-Abl in L-selectin shedding from the neutrophil surface

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ARTICLE INFO

Article history:
Submitted 21 October 2010
Revised 6 December 2010
Available online 1 February 2011

(Communicated by M. Lichtman, M.D,
20 September 2010)

Keywords:
Adhesion
Inflammation
Shear stress
Neutrophil activation

ABSTRACT

L-selectin is a key molecule that participates in neutrophil tethering and subsequent rolling. It is cleaved from the surface of neutrophils activated in the presence of lipopolysaccharides, N-formyl-methionine-leucine-phenylalanine (fMLP), or Interleukin-8 (IL-8). We previously showed that L-selectin is also shed from the neutrophil surface during rolling on sialyl Lewis-x coated surfaces in a force-, ADAM-17 sheddase-, and p38 MAP kinase-dependent manner under flow. c-Abl tyrosine kinase is phosphorylated when L-selectin on the surface of neutrophils is cross-linked with anti-L-selectin antibodies. Here, we study the effect of c-Abl inhibition on L-selectin shedding from primary human neutrophils in static conditions following exposure to fMLP, IL-8, and hypotonic buffer and under flow through sialyl Lewis-x coated microtubes. Results indicate that c-Abl inhibition by STI571 significantly affects neutrophil adhesion via L-selectin, by decreasing the average rolling velocity and increasing the flux of rolling cells. The change in surface receptor expression was verified by flow cytometry. Interestingly, other forms of L-selectin shedding induced by fMLP, IL-8 or osmotic swelling were unaffected by STI571 treatment. These findings implicate the c-Abl signaling molecule in regulating L-selectin mechanical shedding in response to shear stress, setting this type of signaling apart from those triggered by the presence of a hypotonic environment, fMLP, or IL-8. This study sheds light on the role of c-Abl in neutrophil adhesion not previously reported in the literature.

Introduction

The capture and rolling of leukocytes along the endothelium are essential steps leading to the firm adhesion, transmigration, and trafficking of leukocytes to wound or infection sites as part of the inflammation cascade [1–3]. L-selectin is a key molecule that participates in neutrophil tethering and subsequent rolling [4]. For example, L-selectin deficient mice showed impaired leukocyte recruitment into the inflamed endothelium, virtually no lymphocyte migration into the lymph nodes, and impaired neutrophil sequestration in the microvasculature of the lungs in the presence of bacterially induced pneumonia [5,6]. While E-selectin and P-selectin are expressed on activated endothelium, L-selectin is constitutively expressed at the tips of microvilli on the neutrophil surface [7,8]. L-selectin binds to Gly-CAM1, CD34, and podocalyxn found on the endothelial surface of high venules in lymph nodes [9–11]. Heparin sulfate also acts as a primary ligand for L-selectin on the inflamed vascular endothelium [12]. In addition, L-selectin interacts with the single O-linked sialyl Lewis x and three adjacent moieties on P-selectin glycoprotein ligand 1 (PSGL-1), a sialomucin expressed on the surface of neutrophils. PSGL-1 acts as the dominant ligand for E- and P-selectin and interacts with L-selectin leading to secondary leukocyte recruitment via leukocyte-leukocyte interactions [13,14].

In addition to playing a mechanical role in mediating neutrophil adhesion, L-selectin also acts as a signal-transducing receptor [15–17]. Unlike E- and P-selectin, L-selectin is cleaved from the leukocyte surface as a result of cellular activation and inflammatory stimuli [18]. L-selectin ligation in neutrophils has been shown to activate cells as measured by increases in Ca^{2+} flux, superoxide generation, increased adhesiveness and activation of the mitogen activated protein kinase (MAPK) and tyrosine phosphorylation pathways [15,19–22]. The downregulation of L-selectin and conformational change of β2 integrins leading to increased binding affinity are characteristic results of neutrophil activation [23,24]. Stimulus by IL-8, PMA, LPS, or fMLP leads to L-selectin shedding and conformational change in β_2 integrins [7,25,26]. TNF-α-converting enzyme (TACE or ADAM-17) was identified as the protease responsible for L-selectin cleavage [27,28]. Crosslinking of L-selectin with anti-L-selectin mAb or stimulation with sialyl Lewis-x under static conditions leads to the activation of a signaling cascade from tyrosine kinase p56lck to Sos, Ras, MAPK, and Rac2 [29,30].

We previously found that L-selectin is shed from the neutrophil surface as a result of the mechanical stimulus stemming from

Abbreviations: ABL1, c-Abl; CML, Chronic myelogenous leukemia; DPBS, Dulbecco's phosphate buffered saline; Gly-CAM1, Glycosylation-dependent cell adhesion molecule 1; IL-8, Interleukin-8; MEKK1, MAP kinase kinase kinase 1; Mac-1, Membrane attack complex type 1; MAPK, Mitogen-activated protein kinase; MLH1, mutL homologue 1; fMLP, N-formyl-Methionine-Leucine-Phenylalanine; PMA, phorbol 12-myristate 13-acetate; PSGL-1, P-selectin glycoprotein ligand 1; RT, Room Temperature; TACE, TNF-α-converting enzyme.

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doi:10.1016/j.bcmd.2010.12.010

neutrophil rolling on a sialyl Lewis-x coated surface under shear flow [31]. This form of L-selectin shedding is dependent on the rate of flow, indicating mechanical force plays a role in L-selectin cleavage from the neutrophil surface. The activity of ADAM-17 during L-selectin shedding under flow has been studied both *in vivo* [32] and *in vitro* [31]. It was also shown that the pharmacological inhibition of either ADAM-17 or p38 MAP kinase was sufficient to prevent mechanically-induced L-selectin shedding [31]. Mice with ADAM-17 conditionally knocked out exhibit a reduction of L-selectin shedding and an increase in neutrophil adhesion to the blood vessel wall [32]. The increase in L-selectin mediated neutrophil adhesion modified the inflammatory response of mice enough to significantly increase the survival rate of those with *Escherichia coli*-mediated peritoneal sepsis [32].

The propagation of signaling instigated by L-selectin cross-linking is dependent on c-Abl, a non-receptor tyrosine kinase [17]. c-Abl has a catalytic domain, polyproline-rich regions and SH2 and SH3 domains that are involved in protein-protein interactions and regulate the kinase activity itself. The C-terminal end has nuclear localization and nuclear export signals, allowing the protein to have activity inside and outside of the nucleus. Additionally, c-Abl has F-actin and G-actin-binding domains that participate in the regulation of actin polymerization [33,34]. Inhibition of c-Abl through the use of either the pharmaceutical inhibitor STI571 (also known as Imatinib methanesulfonate salt, Imatinib mesylate, or trade name Gleevec®) or conditional knockout mice with the gene truncation solely found in the T cell lineage causes a reduction in F-actin polymerization in T cells [35]. Exposure to thermal cycles or L-selectin ligation by monoclonal antibodies on the neutrophil surface leads to a significant increase in c-Abl kinase activity spatially localized near F-actin [16]. The role of c-Abl in propagating cell signaling in neutrophils under shear flow has not been examined. Thus, the purpose of this study was to examine the effect of c-Abl inhibition on L-selectin shedding from neutrophils during activation and under shear flow.

Materials and methods

Reagents

APC-CD62L mAb, clone Dreg-56, specific for human L-selectin and APC conjugated mouse IgG1 isotype control antibody were obtained from Becton Dickinson, San Jose, CA, USA. FITC conjugated mAb for anti-human CD11b clone CBRM1/5 and FITC conjugated mouse IgG1 isotype control were purchased from eBioscience, San Diego, CA, USA. The c-Abl inhibitor STI571, >99% (LC Laboratories, Woburn, MA, USA) was purchased. Ca^{2+} and Mg^{2+} free HBSS (Invitrogen, Camarillo, CA, USA), Ca^{2+} and Mg^{2+} free DPBS (Invitrogen, Camarillo, CA, USA), calcium carbonate (Sigma Chemical Co., St. Louis, MO, USA), endotoxin free water (MO BIO Laboratories, Carlsbad, CA, USA), endotoxin free human serum albumin (Sigma Chemical Co., St. Louis, MO, USA), and low endotoxin (1 ng/mg), essentially globulin-free BSA (Sigma Chemical Co., St. Louis, MO, USA) were used to make buffer solutions for neutrophil isolation and flow experiments.

Neutrophil isolation

Human peripheral blood was collected from healthy adult donors after informed consent. Neutrophils were isolated by centrifugation at 480*g* for 50 minutes at 23 °C in a Marathon 8K centrifuge (Fisher Scientific, Fair Lawn, NJ, USA) using 1-Step Polymorphs (Accurate Chemical and Scientific Corporation, Westbury, NY, USA.) This zonal centrifugation method uses a density gradient to create separate visible layers of plasma, mononuclear cells, neutrophils, and erythrocytes and platelets. The neutrophil layer was extracted and washed twice in Ca^{2+} and Mg^{2+} free HBSS to remove the polymorph, and any remaining red blood cells were lysed by osmotic swelling. Neutrophils were resuspended at a concentration of 1×10^6 cells/mL. Half of the neutrophils were incubated at RT for 30 minutes in 10 μM STI571. These incubation conditions have been previously used to inhibit c-Abl signaling in neutrophils *in vitro* [16,17,36,37], and it falls within the range of the peak plasma concentrations found in patients taking 400 to 800 mg STI571 as part of a chemotherapy regimen [38]. Following the incubation, the cells were resuspended in HBSS containing 0.5% HSA, 2 mM Ca^{2+}, 10 mM HEPES, buffered to 7.4 with or without 10 μM STI571.

Neutrophil activation under static conditions

Isolated neutrophils were treated with STI571 and incubated with IL-8 or fMLP (R&D Systems Inc., Minneapolis, MN, USA) to determine the effect of STI571 treatment on L-selectin shedding during neutrophil activation under static conditions. IL-8 was dissolved at a concentration of 100 μg/mL in endotoxin free water. fMLP was dissolved at a concentration of 100 μM in DMSO. Both STI571-treated and untreated neutrophils were suspended at a concentration of 1×10^6 cells/mL in HBSS containing 0.5% HSA, 2 mM Ca^{2+}, 10 mM HEPES, buffered to 7.4. Cells were then incubated in either 1 nM IL-8 or 5 nM fMLP for 2 minutes at RT or in 0.5× $Ca^{2+}$and Mg^{2+} free HBSS at RT for 30 minutes. Control samples were treated with equivalent volumes of endotoxin free water or DMSO. Neutrophils were then labeled with anti-L-selectin and CBRM1/5 antibodies at 4 °C, washed with cold $Ca^{2+}$and Mg^{2+} free DPBS, and fixed in cold 4% paraformaldehyde for 30 minutes before analysis by flow cytometry as described below. Experiments were conducted using neutrophils from at least three different donors.

Microtube preparation

Polyurethane microtubes with an inner diameter of 300 μm and external diameter of 600 μm (Braintree Scientific Inc., Braintree, MA, USA) were cut to a length of 50 cm. Two tubes were prepared by drawing up 200 μg/mL NeutrAvidin biotin-binding protein (Thermo Fisher Scientific Inc., Rockford, IL, USA) with insulin needle syringes (Becton Dickinson, San Jose, CA, USA Biosciences) followed by an overnight incubation at 4 °C. Next, the tubes were incubated with 20 μg/mL sialyl Lewis-x-PAA-biotin (GlycoTech Corporation, Gaithersburg, MD, USA) for 2 hours at room temperature (RT). Finally, the tubes were incubated with 1% BSA at RT for 1 hour to block non-specific adhesion. Two BSA control tubes were incubated with 1% BSA for 1 hour at RT.

Microtube flow experiment

Coated microtubes were mounted on an inverted microscope, Olympus IX81 (Olympus America Inc., Melville, NY, USA). Neutrophils were perfused through the microtubes using a syringe pump at a wall shear stress of 1.5 dyne/cm^2. Untreated and STI571-treated neutrophils were perfused through either sialyl Lewis-x coated- or (control) BSA coated microtubes. Videos were recorded for 30 seconds at 10 random locations along the length of each microtube after 5 minutes of neutrophil perfusion. After 30 minutes of flow, HBSS containing 0.5% HSA and 10 mM HEPES buffered to 7.4 was used to collect the remaining neutrophils from the microtubes.

Data acquisition

Videos of the rolling neutrophils were recorded using a microscope-linked Hitachi CCD camera KP-M1AN (Hitachi, Japan) and a Sony DVD Recorder DVO-1000MD (Sony Electronics Inc., San Diego, California, USA). DVD chapters were converted to 640×480 pixels at 30 fps using FFMPEGX (Fabrice Bellard, France). Rolling flux, rolling velocity and detachment rate were determined using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA). Rolling cells were defined as any cell