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Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils

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ABSTRACT

Neutrophil infiltration is a prominent feature in a number of pathologic conditions affecting horses including recurrent airway obstruction, ischemia-reperfusion injury, and laminitis. Cell signaling components involved in neutrophil migration represent targets for novel anti-inflammatory therapies. In order to migrate into tissue, neutrophils must respond to chemoattractant signals in their external environment through activation of adhesion receptors (*i.e.* integrins) and reorganization of the actin cytoskeleton. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS), a highly conserved actin-binding protein, has a well demonstrated role in cytoskeletal dependent cellular functions (*i.e.* adhesion, spreading, and migration), but the details of MARCKS involvement in these processes remain vague. We hypothesized that MARCKS serves as a link between the actin cytoskeleton and integrin function in neutrophils. Using a MARCKS-specific inhibitor peptide known as MANS on equine neutrophils *in vitro*, we demonstrate that inhibition of MARCKS function significantly attenuates β2-integrin-dependent neutrophil functions including migration, adhesion, and immune complex-mediated respiratory burst. The MANS peptide did not, however, inhibit the β2-integrin-independent PMA mediated respiratory burst. These results attest to the essential role of MARCKS function in regulating neutrophil responses, and strongly implicate MARCKS as a potential regulator of β2-integrins in neutrophils.

1. Introduction

Although they are essential for normal host defense, neutrophils feature prominently in the pathophysiology of a number of important equine diseases, including laminitis, ischemia-reperfusion injury and recurrent airway obstruction (Moore et al., 1995; Gerard et al., 1999; Little et al., 2005; Marinkovic et al., 2007; de la Rebiere de Pouyade and Serteyn, 2011). Mechanisms of neutrophil-mediated tissue injury include release of proteolytic enzymes and production of reactive oxygen species (Wong et al., 2012). Despite ample research efforts directed toward understanding neutrophil recruitment, activation, and mechanisms of injury, clinically

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applicable treatments for neutrophil-mediated diseases remain limited.

Neutrophils in systemic circulation are recruited to sites of tissue infection or inflammation by host- or bacterial-derived chemoattractants; first adhering to the luminal surface of post-capillary venules, then moving across the endothelium, and finally crawling through the extracellular matrix to their final destination (Colditz, 1985; Baggiolini, 1998). To accomplish this arduous journey, neutrophils must recognize, interpret, and physically migrate along a chemokine gradient through a process known as chemotaxis. Neutrophil chemotaxis is a complex process that requires coordinated participation of essential cell surface receptors, a hierarchy of secondary cell signaling molecules and dynamic restructuring of the actin cytoskeleton (Dillon et al., 1988; Foxman et al., 1997; Cicchetti et al., 2002). Thus, inhibition of cellular regulators of neutrophil chemotaxis could be utilized to prevent or minimize unwanted neutrophil accumulation in tissues, and are therefore potential targets for novel anti-inflammatory therapies.

In order to move from the vasculature to sites of tissue inflammation, neutrophils must adhere to, and crawl along, inflamed endothelium *via* an integrin-dependent process (Kurtel et al., 1992). Integrins are transmembrane receptors that consist of non-covalently bound heterodimers of α and β chains. While neutrophils express several integrin heterodimers from the β1, β2 and β3 families, intraluminal adhesion and migration are dependent on activation of β2-integrins specifically (Schmidt et al., 2013). There are three key steps to activation of β2-integrins in neutrophils. (1) Increased surface expression of β2-integrins is achieved when secretory vesicles, which contain high numbers of preformed β2-integrins on their membranes, fuse with the neutrophil plasma membrane during exocytosis. (2) Intermediate and high-affinity conformations of β2-integrins are induced by chemoattractant binding to G-protein coupled receptors ("inside-out" signaling) or by direct integrin-ligand binding ("outside-in" signaling). (3) Increased binding avidity occurs when integrins are released from their cytoskeletal constraints and are able to diffuse throughout the cell membrane, resulting in formation of clusters (Nishida et al., 2006; Schymeinsky et al., 2007).

While many of the signaling details regulating integrin affinity and avidity remain unclear, PKC-mediated release of cytoskeletal constraints is known to play a key role in β2-integrin activation (Springer, 1990; Hynes, 1992; Clark and Brugge, 1995; Rosales and Juliano, 1995; Zhou and Li, 2000; Larsson, 2006). As a prominent PKC substrate and actin-binding protein, the MARCKS protein (Myristoylated Alanine Rich C-Kinase Substrate) has been proposed as a key link between PKC, actin, and integrin molecules (Aderem, 1992; Hartwig et al., 1992a; Blackshear, 1993; Arbuzova et al., 2002). Indeed, previous research from our laboratory has demonstrated that inhibition of MARCKS function attenuates the β2-integrin-dependent processes of migration and adhesion in human neutrophils *in vitro* (Eckert et al., 2009). In the current study, our goal was to further investigate the potential link between β2-integrin-dependent neutrophil functions and MARCKS. To this end, we measured the β2-integrin-dependent neutrophil functions of migration, adhesion and respiratory burst *in vitro*, with and without MARCKS inhibition. These data were compared to results from similar experiments conducted with or without β2-integrin-specific inhibition. Equine neutrophils were utilized in order to gain comparative species data to complement our previous study, to expand on our previous results with a human-relevant animal model, and to conduct research relevant to veterinary species, as well as humans.

To block MARCKS function, we utilized the MARCKS-specific inhibitor peptide known as "MANS" (myristoylated n-terminal sequence) as previously described (Singer et al., 2004; Takashi et al., 2006; Eckert et al., 2009; Li et al., 2013, Ott et al., 2013). RNS (random n-terminal sequence), which is a scrambled version of the same 24 amino acids as MANS, was used as a control. To block β2-integrin function we inhibited the integrin β chain (CD18) with the $F(ab^1)_2$ portion of an anti-CD18 antibody (αCD18). Interestingly, these results show that inhibition of β2-integrin (using αCD18) or MARCKS (using 50 μM MANS) attenuates equine neutrophil migration, adhesion and respiratory burst to a similar degree. Our findings also demonstrate that MARCKS is essential for β2-integrin-dependent neutrophil functions, but is *not* essential for β2-integrin-independent functions (*i.e.* PMA-mediated respiratory burst) in equine neutrophils. Taken together, these results strongly suggest that MARCKS function is essential to β2-integrin-dependent processes in neutrophils. Studies are currently underway to determine which aspects of integrin activation and/or signaling are dependent on MARCKS function. Our findings support the assertion that inhibitors of MARCKS deserve further study as potential therapies for neutrophil mediated tissue injury.

2. Materials and methods

2.1. Donors and neutrophil isolation

Animal use protocols were reviewed and approved by the North Carolina State University IACUC review board. For all neutrophil experiments, 30–60 ml of whole blood was collected using heparinized syringes from the jugular vein of adult horses. As healthy members of the teaching animal unit herd at NCSU College of Veterinary Medicine, all donors were fed and housed under the same conditions and were receiving no medical treatment at the time of blood collection. Neutrophils were isolated from whole blood using Ficoll-Paque™ Plus (GE Healthcare, Sweden) density gradient centrifugation (Nauseef, 2007). Briefly, heparinized whole blood was aliquoted into 15 ml polypropylene conical tubes (Sarstedt) and allowed to settle at room temperature for 45–60 min. Up to 10 ml of leukocyte rich plasma was aspirated using a bulb syringe and layered on 5 ml of Ficoll in a separate 15 ml conical tube. Cells were then centrifuged at 1800 rpm for 20 min. The supernatant was discarded and remaining red blood cells within the cell pellet were removed by 60 s of hypotonic lysis. Isolated neutrophils (>96% by Wright's Geimsa staining) were resuspended/washed in sterile HBSS (Cellgro, Inc.) without additives. Cell number and viability was