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Research paper

EB1 contributes to proper front-to-back polarity in neutrophil-like HL-60 cells

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ABSTRACT

Directed migration of leukocytes towards a chemotactic source is largely dependent on coordinated actin cytoskeleton functions that provide the driving forces at the cell front and enable contractility at the rear. In contrast to the force-generating properties of the actin cytoskeleton, the microtubule network assumes a regulatory function in balancing front-to-back polarity. In migrating neutrophils, microtubules are mostly concentrated at the cell rear, and previously published work suggested that microtubules are stabilized and kept in place by a mechanism involving Cdc42, WASP, CD11b, and the end-binding protein 1 (EB1). EB1, as a microtubule plus-end tracking protein (+TIP), is a potential candidate to bridge the gap between microtubule and actomyosin dynamics. After knockdown of EB1 in neutrophil-like HL-60 cells, both directionality and straightness of migration while moving through 3D collagen gels are impaired. An increased number of lateral protrusions are observed in EB1-knockdown cells, indicating an inability to balance cell polarity in the absence of EB1. Moreover, in EB1-deficient cells, substrate adhesion on fibrinogen-coated surfaces is significantly reduced. EB1-knockdown cells show significant changes in levels of GEF-H1, a microtubule-associated guanine nucleotide exchange factor that links microtubule integrity to RhoA-dependent regulation of the actin cytoskeleton, suggesting that GEF-H1 might constitute one element of the microtubule-actin crosstalk in migrating leukocytes.

1. Introduction

Directional cell migration plays a fundamental role for a variety of processes, such as morphogenesis, wound healing, and host defence. During innate immune responses, leukocytes interpret gradients of inflammatory cues or stimuli of pathogenic origin in order to find and migrate toward the site of infection and kill invading bacteria. During this multistep process, blood neutrophils, which constitute the most abundant leukocytes of the human body, tether, roll, adhere, spread and crawl on the vessel wall before transmigrating through the endothelium into infected tissue (Kruger et al., 2015; Muller, 2013; Voisin and Nourshargh, 2013). Within the tissue, directional migration is an active process that is driven by highly polarized and dynamic rearrangements of the neutrophils' actin cytoskeleton (Germena and Hirsch, 2013). When moving on plain substrate, protrusive forces propelling the leading edge forward are provided by polymerization of actin at the cell front, whereas actomyosin-based contractility at the uropod prevents pseudopod formation at the rear, thereby increasing the persistence of movement along a gradient.

For forward locomotion in a two-dimensional (2D) environment, neutrophils need to gain traction on the substrate. This is achieved by integrins, which specifically bind to a broad range of ligands like extracellular matrix (ECM) proteins and counter-receptors exposed by the vascular endothelium (Pick et al., 2013). However, neutrophils can move in a protrusive manner through three-dimensional (3D) matrices, such as the diverse microenvironments of the interstitium, without integrin-based adhesion, just by transmission of forces generated by actin polymerization on the encompassing fibrils and surfaces (Lämmermann et al., 2008; Lämmermann and Germain, 2014; Nourshargh et al., 2010).

While, with very few exceptions, the actin cytoskeleton ultimately provides the driving forces for locomotion, microtubules assume a crucial regulatory function in coordinating persistent

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directed migration. The organization of microtubules and their role in this process differs fundamentally between slow-moving cells, most prominently fibroblasts or tumour cells, and fast-moving cells like neutrophils (Eddy et al., 2002; Kaverina and Straube, 2011; Li and Gundersen, 2008; Niggli, 2003). In slow-moving cells, microtubules predominately radiate towards the leading edge, where they are selectively stabilized and anchored to the cortex by a complex of Diaphanous-related formins, the microtubule plus-end tracking protein (+TIP) EB1 (end-binding protein 1), and Adenomatous polyposis coli (APC) protein (Bartolini et al., 2008; Palazzo et al., 2004; Wen et al., 2004). EB proteins are considered as master regulators of the microtubule +TIP network, but other proteins, such as CLASPs, have been shown to modulate EB localization at microtubule tips (Grimaldi et al., 2014), or to coordinate actin-microtubule interaction via EB1 as shown for spectraplakins (Suozzi et al., 2012). Experimental depolymerization of microtubules in slow-moving cells was shown to result in loss of polarity and entirely halted cell migration.

Microtubule organization in polarized neutrophils seems to depend on the surrounding environment. While moving haptokinetically on plain substrate, the centrosome was reported to localize behind the nucleus or within the lobes (Eddy et al., 2002), whereas in neutrophils of zebrafish embryos moving through 3D tissue, the centrosome is located in front of the nucleus just behind the leading edge (Yoo et al., 2012). In both cases, and unlike slow-moving cells, the majority of microtubules radiate towards the cell rear. The current view is that by confining RhoA-mediated 'backness' signalling to the cell rear, microtubules act as a buffer system to balance proper front-to-back polarity in migrating neutrophils (Ku et al., 2012; Xu et al., 2005). Disturbance of this system causes excess of either 'frontness' or 'backness' signalling, and leads to defective polarization and inaccurately or weakly defined cell fronts. This crosstalk between microtubules and the actomyosin system might, at least partially, be mediated by the Rho/Rac guanine nucleotide exchange factor 2 (GEF-H1), which is regulated through binding to microtubules. While its microtubule-bound form is inactive, GEF-H1 is activated upon release and triggers an increase in GTP-RhoA levels. Among other effectors, GTP-RhoA activates p160-ROCK to enhance actomyosin contractility (Birkenfeld et al., 2008; Krendel et al., 2002).

By undergoing dynamic instability, the microtubule plus end determines the dynamic state of a microtubule (Akhmanova and Steinmetz, 2015). The central molecules of growing microtubule tips are the EB proteins, in particular EB1 (Akhmanova and Steinmetz, 2010, 2015). As homo- or hetero-dimers, EB proteins autonomously associate with growing microtubule ends and provide binding sites for more than 15 other +TIP proteins (Bieling et al., 2007; Galjart, 2010; Kumar and Wittmann, 2012). Although a significant number of studies have been conducted, the exact impact of EB1 on microtubule dynamics is still not entirely clear. *In vitro*-studies have suggested either a stabilizing (Komarova et al., 2009; Manna et al., 2008; Maurer et al., 2011; Vitre et al., 2008), or destabilizing effect on microtubules (Komarova et al., 2009; Vitre et al., 2008). In analogy to its ability to capture and stabilize microtubules at leading edges of fibroblasts, EB1 may also contribute to microtubule stabilization in neutrophils. It has been shown recently that upon stimulation of neutrophils with cytokines, CD11b is rearranged in a Cdc42- and WASP-dependent manner and clustered into specific membrane domains at the uropod, where it captures and anchors microtubules via binding to EB1 (Kumar et al., 2012; Szczur et al., 2009). As a consequence, these microtubules are stabilized and corresponding tubulin dimers are detyrosinated (Glu-MT), further lowering their dynamicity (Kumar et al., 2012; Song and Brady, 2015; Westermann and Weber, 2003). Ultimately, in neutrophils this leads to an organization with only few dynamic microtubules radiating towards the cell front, whereas the majority of microtubules orient towards the back.

Previous studies have elegantly illustrated the importance of microtubules for neutrophil chemotaxis on plain substrate, however, only little is known about their contribution to movement in 3D environments (Eddy et al., 2002; Ku et al., 2012; Niggli, 2003; Xu et al., 2005; Yoo et al., 2012). Here, we provide evidence that during locomotion through 3D collagen gels, microtubules act by stabilizing the directionality of migration, thereby increasing the efficiency of chemotaxis. Given the generally different mode of locomotion and the divergent microtubule organization within 3D environments, this result was by no means apparent. Furthermore, we found that EB1-knockdown cells display impaired directionality of chemotaxis when moving through 3D collagen gels, similar to cells with disrupted microtubules. Moreover, when adhesion and polarization was induced on plain substrates, EB1-knockdown cells exhibited a substantial increase of abnormally polarized cells lacking leading edges. We suggest that the EB1-knockdown phenotype manifests via misregulation of GEF-H1 activity, possibly due to alterations of microtubule dynamics.

2. Materials and methods

2.1. Generation of vectors

The gene coding for human (h) EB1 (UniGene Cluster ID HS.472437) was acquired from Source Bioscience (Nottingham, UK), amplified by PCR using primers cgcGGTACCatggcagtgaacgtatactcaac (f)/cgcGGATCCttaatactcttcttgctcctcctg (r), and cgcCTCGAGatggcagtgaacgtatactcaac (f)/cgcGGATCCgaatactcttcttgctcctcctg (r), respectively, and cloned into pEGFP-C1 and pEGFP-N1 (Clontech, Mountain View, CA, USA) via *Kpn*I and *Bam*HI. hEB2-GFP (transcription variants 1 and 2) were cloned via *Hin*dIII and *Sal*I restriction sites using forward primers gcgAAGCTTGCatgcctgggccgac and gcgAAGCTTGCatggcggtcaatgtgtatt, respectively, in combination with reverse primer gcgGTCGACtcagtactcttcctgctgcg.

The hCLIP-170-GFP expression vector was obtained by performing a three-fragment ligation comprising a 5′-PCR fragment (primers gcgCTCGAGGCatgagtatgctaaagccaagtg (f) and gactcgttctctttggaaagc (r)) cut with *Xho*I/*Acc*I, a 3′-PCR fragment (primers gaaactcatcagaaggagataaagg (f) and gcgGAATTCtcagaaggtttcgtcgtca (r)) cut with *Eco*RI/*Acc*I, and pEGFP-N1 vector cut with *Xho*I/*Eco*RI.

Templates for PCR reactions on cDNA were obtained by performing RT-PCR on purified total RNA extracted from non-differentiated or differentiated HL-60 cells using a reverse transcriptase Kit (Roche Diagnostics, Penzberg, Germany), and PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA), respectively.

2.2. Cell culture and transfection of HL-60 cells

HL-60 cells (ATCC, Manassas, VA, USA) were cultivated in RPMI-1640 medium containing HEPES and sodium bicarbonate (R5886, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) at 37° C in a humidified atmosphere containing 5% CO_2. Differentiation to a neutrophil-like state was induced by cultivation in RPMI-medium containing 1.3% DMSO for 5–6 days.

Transient transfection of HL-60 cells was achieved employing an Amaxa Nucleofector II device with Kit T reagents and program T-19 (Lonza, Basel, Switzerland). To generate stable HL-60 knockdown cells as described previously (Schymeinsky et al., 2009), HL-60 cells were transduced with vectors containing shRNA specific for hEB1 (MISSION shRNA Library SHCLND-NM_012325 comprising