Roles of Rho GTPases in leucocyte and leukaemia cell transendothelial migration

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Leucocytes migrate into and out of blood vessels at multiple points during their development and maturation, and during immune surveillance. In response to tissue damage and infection, they are rapidly recruited through the endothelium lining blood vessels into the tissues. Leukaemia cells also move in and out of the bloodstream during leukaemia progression. Rho GTPases are intracellular signalling proteins that regulate cytoskeletal dynamics and are key coordinators of cell migration. Here, we describe how different members of the Rho GTPase family act in leucocytes and leukaemia cells to regulate steps of transendothelial migration. We discuss how inhibitors of Rho signalling could be used to reduce leucocyte or leukaemia cell entry into tissues.

1. Introduction

Rho GTPases regulate cytoskeletal dynamics, and thereby affect a variety of cellular processes including cell polarity, migration, adhesion and vesicle trafficking. Rho GTPases are highly conserved in evolution and found in nearly all eukaryotes sequenced to date. There are 20 Rho GTPase genes in humans, most of which encode proteins of 20–28 kDa. Rho GTPase expression varies across tissues and is regulated by multiple stimuli. They exert their function by interaction with downstream targets including protein kinases, phospholipases, adaptor proteins and actin nucleators [1,2].

Most Rho GTPases cycle between a guanine diphosphate (GDP)-bound inactive form and a guanine triphosphate (GTP)-bound active form that interacts with downstream effector proteins. The GTP–GDP switch is regulated by guanine nucleotide exchange factors (GEFs) that promote exchange of bound GDP for GTP and GTPase activating proteins (GAPs) that stimulate GTP hydrolysis to GDP. The Rho GTPases RhoU and RhoV have a high-intrinsic GDP/GTP exchange rate and are therefore mainly GTP-bound in cells [3]. Rnd, RhoH and RhoBTB proteins lack GTPase activity, hence they are constitutively bound to GTP [4].

Rho GTPases are post-translationally modified by addition of isoprenyl and/or palmitoyl lipid groups at the C-terminus, facilitating their interaction with membranes where they signal to downstream effectors [5]. Unlike isoprenylation, palmitoylation is a reversible process. This allows proteins to associate transiently with membranes, regulating their localization or endocytic trafficking [6,7]. The Rho GTPases RhoU and RhoV are targeted to membranes by palmitoylation and are not prenylated. Rac1 was recently demonstrated to be palmitoylated, which requires its prior prenylation. Rac1 palmitoylation appears to be important for it to stimulate actin cytoskeleton remodelling [8].

The Rho GTPase Cdc42 is well known to contribute to cell polarity in multiple eukaryotic organisms, from polarized growth and mating in yeasts, to polarized cell migration and epithelial polarity in mammals [9]. Here, we focus on the roles of Rho GTPases in leucocytes and leukaemia cells during their polarized migration across the endothelium.

2. Rho GTPases and leucocyte migration

Cell polarization and migration are driven by actin cytoskeletal dynamics, which is regulated by Rho GTPases acting on actin nucleators and actin-regulatory proteins.
Migrating leucocytes have an F-actin-rich lamellipodium at the front and a contractile uropod at the back (figure 1). Transmigrating leucocytes extend lamellipodia and filopodia under the endothelium [10] (figure 2). The main regulators of lamellipodium extension in leucocytes are Rac1 and Rac2 [11,12]. Rac GTPases regulate actin polymerization through the WAVE complex, consisting of WAVE, PIR121/Sra-1, Nap125, HSPC300 and Abl interactor. Rac binds to PIR121/Sra-1. In response to stimuli, WAVE can activate the Arp2/3 complex, inducing actin polymerization [13]. Cdc42 can also contribute to membrane extension via WASP which activates the Arp2/3 complex. RhoA is active at the front of the cell and is proposed to induce actin polymerization and membrane protrusion via the formin mDia and membrane retraction via ROCK. Cdc42 induces filopodia via mDia. RhoA activation at the rear of the cell controls uropod contractility through ROCK inhibition of MLC phosphatase and subsequent MLC phosphorylation. In polarized leucocytes, the microtubule organizing centre (MTOC) normally localizes behind the nucleus in the uropod region. (Online version in colour.)

Figure 1. Leucocyte polarity and Rho GTPases. Rac1 activates the WAVE complex, leading to Arp2/3-mediated actin polymerization at the leading edge to form a lamellipodium. Cdc42 can also contribute to membrane extension via WASP which activates the Arp2/3 complex. RhoA is active at the front of the cell and is proposed to induce actin polymerization and membrane protrusion via the formin mDia and membrane retraction via ROCK. Cdc42 induces filopodia via mDia. RhoA activation at the rear of the cell controls uropod contractility through ROCK inhibition of MLC phosphatase and subsequent MLC phosphorylation. In polarized leucocytes, the microtubule organizing centre (MTOC) normally localizes behind the nucleus in the uropod region. (Online version in colour.)

Figure 2. Roles of Cdc42, Rac1/2 and RhoA in transendothelial migration (TEM). Schematic of leucocyte TEM. Rolling is the first step of TEM, which is mediated by selectins and their ligands. Subsequently, leucocytes firmly adhere to the endothelium through binding of active β1 and β2 integrins to VCAM-1 and ICAM-1 receptors on the endothelium. Leucocytes can then transmigrate via a paracellular route passing through endothelial junctions or a transcellular route through a transient pore in the body of an endothelial cell. The Cdc42 target WASP affects leucocyte adhesion by regulating β2 integrin clustering. Cdc42, Rac1/2 and RhoA all affect the ability of leucocytes to polarize and migrate on top of endothelial cells. RhoA acts at the front and back of transmigrating cells during TEM, and Rac1/2 also contribute to TEM. (Online version in colour.)

[2]. Migrating leucocytes have an F-actin-rich lamellipodium at the front and a contractile uropod at the back (figure 1). Transmigrating leucocytes extend lamellipodia and filopodia under the endothelium [10] (figure 2). The main regulators of lamellipodium extension in leucocytes are Rac1 and Rac2 [11,12]. Rac GTPases regulate actin polymerization through the WAVE complex, consisting of WAVE, PIR121/Sra-1, Nap125, HSPC300 and Abl interactor. Rac binds to PIR121/Sra-1. In response to stimuli, WAVE can activate the Arp2/3 complex, inducing actin polymerization [13]. Cdc42 can also activate WASP and N-WASP leading to actin nucleation by the Arp2/3 complex [14].

Formin family proteins nucleate non-branching actin filaments and also contribute to lamellipodium as well as filopodium formation [15,16]. Depolymerization of actin filaments is mediated by ADF/cofilin proteins that bind preferentially to ADP-actin, promoting the release of actin monomers. Cofilin activity is regulated by Rac and Rho via PAK and ROCK, which phosphorylate LIMK, which in turn phosphorylates and inhibits cofilin [17]. Cdc42 induces
filopodium formation via mDia formins; transmigrating T-acute lymphoblastic leukaemia (T-ALL) cells extend filopodia under the endothelium [13].

The uropod at the back of migrating leucocytes contains contractile actomyosin filaments, which require RhoA and ROCK for their assembly [18] (figure 1). Actin filaments are linked to the plasma membrane and transmembrane receptors in the uropod by ezrin–radixin–moesin proteins, which are also linked to Rho/ROCK signalling.

### 3. Leucocyte and leukaemia cell transendothelial migration

Leucocytes cross the endothelium in low numbers into lymph nodes and tissues during immune surveillance [19]. At a site of inflammation, however, endothelial cells get activated and upregulate expression of adhesion receptors for leucocytes including selectins, ICAM-1 and VCAM-1 [20]. This leads to rapid recruitment of leucocytes into inflamed tissues. Leukaemia cells proliferate in niches in the bone marrow and/or lymph nodes, and therefore need to adhere to and cross the endothelium to enter these sites [21–23].

Leucocytes first roll on endothelial cells through low-affinity interactions between selectins and selectin ligands. Chemokines present on the surface of activated endothelial cells then stimulate integrin activation on leucocytes, leading to firm adhesion to ICAM-1 and VCAM-1, followed by transendothelial migration (TEM) [24] (figure 2).

Leucocytes can cross the endothelium by going between endothelial cells (paracellular route), which involves local disassembly of endothelial cell–cell junctions [25] (figure 2). Alternatively, they can take a transcellular route, in which a transcellular pore through an endothelial cell transiently opens, allowing the leucocyte to cross through [26]. The choice between these two routes may depend on the tissue, endothelial stimulus and type of leucocyte.

### 4. Cdc42 and transendothelial migration

Cdc42 was found to prevent the formation of a lamellipodium in the uropod, thereby preventing the formation of ‘two-headed’ T cells and preserving polarity [27]. Cdc42 is required for chemokine-driven TEM of effecter memory CD4 T cells [28]. However, T-ALL cells do not show impairment of TEM following Cdc42 depletion [10].

The Cdc42 effector WASP contributes to TEM. WASP-deficient macrophages and neutrophils show defective clustering of β2 integrins (which bind to the endothelial receptor ICAM-1) and impaired adhesion to and transmigration across endothelial cells [29]. Another effector for Cdc42 is Par6, which is part of the polarity complex that includes Par3 and the atypical PKCs (aPKCz and aPKCζ). The Par complex controls T cell migratory polarity [30], but so far it is not known whether Par3, Par6 and aPKCs contribute to TEM.

### 5. Rac proteins and transendothelial migration

The two main Rac proteins expressed in leucocytes, Rac1 and Rac2, appear to have different functions. Rac2 depletion reduces T-ALL cell TEM, whereas Rac1 depletion had only a small effect on TEM [10]. This might reflect a higher level of Rac2 in T-ALL cells. However, in neutrophils, Rac1 and Rac2 contribute different aspects of actin dynamics in lamellipodia [31–33].

Interestingly, the RacGEF Tiam1 controls the route of T cell TEM: Tiam1-deficient mouse T cells predominantly use a transcellular route rather than paracellular route. PKCζ, Tiam1 and Rac are required for efficient migration of T cells on endothelial cells [34]. As Rac-driven lamellipodia are likely to be required for both paracellular and transcellular leucocyte TEM, this suggests that different RacGEFs are involved for transcellular versus paracellular TEM, presumably because different leucocyte receptors mediate each pathway.

### 6. RhoA regulates leucocyte polarity on endothelial cells

The first evidence that Rho subfamily members in leucocytes contributed to TEM came from studies using the Clostridial exoenzyme C3 transferase, which covalently ADP-ribosylates and inhibits the closely related proteins RhoA, RhoB and RhoC [35]. Monocytes treated with C3 transferase adhered normally to endothelial cells but showed a defect in tail retraction, preventing them from completing TEM.

We subsequently showed that RhoA plays a central role both at the leading edge and uropod of transmigrating T-ALL cells. Active RhoA was found at the front and back of T-ALL cells during TEM and its activity correlated with both retraction and protrusion [10]. At the front, it colocalized with the formin mDia1, suggesting that it could stimulate actin polymerization in lamellipodia and/or filopodia extending under endothelial cells (figure 2). It was also associated with localized areas of retraction in the lamellipodium, suggesting that it could be important for determining turning.

The uropod generates a contractile force through myosin II interaction with actin bundles [13]. RhoA is a major player in the T cell uropod because it is a regulator on myosin II activity. RhoA induces myosin light chain (MLC) phosphorylation and inhibits MLC phosphatase via its downstream targets ROCK1 and ROCK2 [36,37]. The RhoA/ROCK/myosinII axis was also shown to be important in T-ALL TEM by controlling actomyosin contractility, and indeed knock-down of both ROCK1 and
These data suggest that RhoU affects migration of T-ALL cell lines, suggesting that Notch1 siRNA depletion induced a decrease in the adhesion possibly through its role in adhesion. It was also shown that RhoU was demonstrated to be important for T-ALL cell adhesion to fibronectin and also for chemotaxis and migration, than GTP/GDP cycling [4].

RhoH, which are atypical Rho GTPase family members that are predominantly GTP-bound and regulated by changes in phosphorylation, gene expression and degradation, rather than GTP/GDP cycling [4].

RhoH suppresses β2 integrin/LFA-1 adhesion to ICAM-1 and impairs chemotaxis [38,39], so would be expected to reduce TEM of T cells (figure 3), although this has not been tested. Homing of CLL cells lacking RhoH to the bone marrow is reduced [40], which might, in part, reflect a decrease in interaction with bone marrow endothelial cells.

RhoU was described as a target gene for the Notch1 oncogene in T-ALL [41]. RhoU is most closely related to Cdc42 (57% identical at the amino acid sequence level) but together with Rac1 and Rac2 and Cdc42 have been implicated in leucocyte migration in vivo, although so far their roles in TEM have not specifically been studied. These include RhoU and RhoH, which are atypical Rho GTPase family members that are predominantly GTP-bound and regulated by changes in phosphorylation, gene expression and degradation, rather than GTP/GDP cycling [4].

Several small molecule inhibitors that affect Rho GTPase signalling have been tested for their effects on TEM and have the potential to be used clinically either to treat leukaemia patients or to reduce chronic inflammation. Statins are best known for their effects on cholesterol levels, but the same metabolic pathway leads to synthesis of isoprenyl groups used for Rho family GTPase modification as well as multiple other prenylated proteins [44] (figure 4). Statin treatment of cells reduces Rho family signalling by reducing their interaction with membranes, which are their principal sites of action [45]. Interestingly, statin treatment of T-ALL cells reduces TEM, in part by reducing integrin LFA-1 activation and adhesion to its ligand ICAM-1. It turns out that the effect of statin treatment on T-ALL adhesion is mediated by Rap1b [46]. However, RhoA and Rac1 are also affected by statins, and it is likely that they contribute to the reduction in TEM in statin-treated T-ALL cells, but not specifically to LFA-1 activation.

Further evidence for Rac and Rho signalling in TEM comes from treatment of cells with the Rac inhibitor NSC23766 and ROCK inhibitors. The Rac inhibitor appears to reduce Rac activation by affecting GEF interaction with Rac [47]. It would be predicted to reduce TEM although this has not so far been directly tested. The ROCK inhibitor Y-27632 was initially shown to inhibit TEM of monocytes by inhibiting tail retraction [35]. Y-27632 also inhibited tail retraction of T-ALL cells migrating on endothelial cells [10]. In vitro, the ROCK inhibitor fasudil reduced leucocyte interaction with endothelial cells following ischaemia–reperfusion [48], although the relative contribution of fasudil treatment to endothelial versus leucocyte responses was not established. Fasudil is well known to inhibit endothelial activation [49], and thus more studies on how it contributes to leucocytes and leukaemia cells during TEM in vivo would be interesting.
9. Conclusions and future perspectives

The classical Rho GTPases RhoA, Rac1/2 and Cdc42 have each been studied in the context of leukocyte and/or leukaemia cell TEM in vitro, but so far little is known about how other members of the Rho family contribute to this process. It will be important to characterize further the roles of each Rho GTPase in the different steps of the TEM process, from rolling, integrin-mediated adhesion, polarization and choice of transcellular versus paracellular routes of transmigration (figure 2). In the future, targeted knock-out of Rho GTPases in leukocytes and leukaemia models combined with intravital microscopy [50] should determine the relative contribution of each family member to TEM in vivo. Finally, identifying the specific RhoGEFs and downstream targets of Rho GTPases involved in TEM should allow the development of more targeted therapies for inhibiting chronic inflammation and leukaemia progression.

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