Rho GTPases and Leukocyte Adhesion Receptor Expression and Function in Endothelial Cells

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Abstract—Rho family GTPases are key signal transducers that regulate cell adhesion and migration and a variety of other cellular responses, including changes in gene expression. In this review, we discuss how Rho GTPases regulate signaling by endothelial cell receptors involved in leukocyte extravasation. First, Rho GTPases affect the expression of some leukocyte adhesion molecules on endothelial cells, such as intracellular adhesion molecule-1 and E-selectin, that can be induced by proinflammatory mediators, hypoxia, or shear stress. Second, Rho GTPases are activated by engagement of several leukocyte adhesion receptors and contribute to both early morphological changes and subsequent alterations in gene expression. Rho GTPases are therefore candidate targets for inhibiting leukocyte transendothelial migration in heart disease and chronic inflammatory disorders. (Circ Res. 2006;98:757-767.)

Key Words: adhesion molecules ■ Rho GTPases ■ leukocyte transmigration ■ diapedesis ■ endothelial receptors

The endothelium regulates the transport of solutes, proteins, and cells between the blood and the interstitial space. It plays a key role in physiological processes such as innate and adaptive immune responses, and its dysfunction is associated with the development of pathologies such as atherosclerosis and other cardiovascular disorders. Inflammatory and immune responses themselves also contribute to the development of cardiovascular disease.1,2 Leukocyte transmigration across the endothelial barrier, known as transendothelial migration (TEM) or diapedesis, is pivotal to the inflammatory response. Leukocytes cross the endothelial barrier in a multistep process involving the capture and rolling of leukocytes on the blood vessel wall, firm adhesion of leukocytes to endothelial cells (ECs), and subsequent leukocyte crawling and transmigration.3–5 Proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and the lipopolysaccharide (LPS) of bacterial walls, or hemodynamic forces imposed by blood flow increase the surface levels of a variety of molecules on ECs implicated in EC–leukocyte interaction.6–8 Moreover, both these stimuli and EC–leukocyte interaction induce changes in endothelial cell shape, permeability, and gene expression.9–12
Rho GTPases regulate cytoskeletal organization and cell adhesion, thereby contributing to cell migration and endothelial permeability. In addition, it is now well established that Rho GTPases affect gene expression. Here, we review how Rho GTPases contribute to EC-leukocyte interaction, first by regulating the expression of leukocyte adhesion receptors on ECs, and second by transducing signals from these receptors leading to changes in cell morphology and gene expression.

Leukocyte TEM Involves Several Endothelial Receptors

Leukocyte TEM is regulated by the cooperative action of adhesion molecules on both the EC and the leukocyte. Leukocytes first establish transient interactions with the endothelium that allow them to roll along the endothelial surface. This is achieved primarily through the interaction of members of the selectin family and their ligands. Leukocytes then encounter chemokines on the endothelial surface. This activates leukocyte integrins such as αLβ2 (also known as lymphocyte function-associated antigen-1; LFA-1) and α4β1 (also known as very late antigen-4; VLA-4), allowing them to establish firm adhesions with the EC by interacting with EC intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). ICAM-1 and VCAM-1 are enriched in F-actin-rich cup-like “docking” structures that extend around leukocytes bound to the apical EC surface and may be important for TEM. Leukocytes cross ECs either through intercellular junctions (paracellular pathway) or through the EC body (transcellular pathway). Paracellular TEM involves platelet-endothelial cell adhesion molecule-1 (PECAM-1) and members of the junctional adhesion molecule (JAM) family. Less is known about the mechanism underlying the transcellular route, which is often observed in vivo but at a low frequency in vitro. Leukocyte-EC interaction is believed to drive cytoskeleton and membrane rearrangements to “open” a transient channel across the EC for leukocyte transcellular migration. Recently, ICAM-1 has been shown to be important for both transcellular and paracellular TEM of neutrophils, and in particular transcellular TEM depends on the ICAM-1 intracellular tail, indicating that ICAM-1-induced signaling is important for this process. Indeed, ICAM-1 engagement rapidly induces changes in EC morphology and redistribution of cell membrane proteins. This is followed by changes in gene expression, including modulation of endothelial adhesion molecule expression and production of proinflammatory mediators, which may mark these sites on the endothelium to recruit further leukocytes and prolong the inflammatory response.

In addition to being regulated by gene expression and cell surface levels, the leukocyte-binding receptors E-selectin, P-selectin, ICAM-1, and VCAM-1 can be regulated by proteolytic cleavage of the extracellular domain. The soluble extracellular domains generated may inhibit leukocyte-EC interactions by binding to and blocking the receptors on the leukocyte surface. They might also have chemoattractant activity.

Rho GTPases: An Overview

The Rho family comprises 22 genes encoding at least 25 proteins in humans, of which the Rho, Rac, and Cdc42 proteins have been studied in the most detail. Within the Rho family, there are subgroups of closely related isoforms, including Rho (A, B, C) and Rac (1, 2, 3). These isoforms are unlikely to be functionally redundant, but unfortunately most studies do not distinguish between them. All Rho family members bind GDP, and most exhibit GTPase activity and cycle between an inactive GDP-bound form and an active GTP-bound form. This cycling is finely regulated by 3 groups of proteins: the guanine nucleotide exchange factors (GEFs) as activators, and the GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs) as negative regulators. When bound to GTP, Rho GTPases interact with their downstream effectors, which include protein kinases, regulators of actin polymerization, and other proteins with adaptor functions. The selective interaction of the different Rho GTPases with a variety of effectors determines the final outcome of their activation. For example, the interaction of the Rho isoforms RhoA, RhoB, and/or RhoC with ROCK family kinases affects actin organization, whereas their interaction with Dia1 stimulates actin polymerization. The p21-activated kinase (PAK) family of proteins acts downstream of both Rac and Cdc42, affecting actin organization and e-Jun N-terminal kinase (JNK) activation, whereas Wiskott-Aldrich syndrome protein (WASP) is activated by Cdc42 and regulates actin dynamics.

Several approaches have been used to study the involvement of Rho GTPases in cellular processes. Expression of dominant-negative or constitutively active forms of RhoA, Rac1, and Cdc42 has been extensively used to implicate a specific Rho GTPase in a particular response. In addition, bacterial toxins that act selectively on certain Rho GTPases are useful tools for investigating Rho GTPase function. For example, the C3-like ADP-ribosyltransferases ADP-ribosylate the Asn41 residue of RhoA, B, and C, blocking their function.

Glucosylation of Thr37 of Rho, or the equivalent Thr35 of Rac/Cdc42/Ras, is catalyzed by various different clostridial toxins and also inhibits Rho GTPase activity. Other toxins have a positive effect in Rho activity, for example cytotoxic necrotizing factors from Escherichia coli deamidate Gln63, and thereby inhibit GTP hydrolysis.

Rho GTPase function is also regulated by their localization. Most Rho family proteins are post-translationally modified by prenylation of a cysteine residue located 4 amino acids from the C-terminus, followed by methylation of this cysteine and proteolytic removal of the last 3 amino acids. The prenyl group helps anchor the protein to membranes. Isoprenoids are intermediates in the pathway to cholesterol synthesis, and their production is reduced by statins, a group of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that have been widely used in the treatment of hypercholesterolemia. In addition to their cholesterol-lowering effect, some of the beneficial effects of statins are due to the inhibition of farnesyl- and geranylgeranyl-pyrophosphate synthesis, the isoprenoids required for prenylation of Rho proteins. Statins thereby modulate Rho family protein subcellular localization, and this
affects their stability and activity. Statins also alter Rho expression at the transcriptional level, although the mechanistic basis for this is not clear.50,51 Some effects of statins, such as the modulation of endothelial nitric oxide synthase (eNOS) expression17 and smooth muscle cell proliferation,52 have been attributed to inhibition of Rho function because similar effects were observed by expressing dominant-negative RhoA or treating cells with Clostridium botulinum C3 transferase.

Rho GTPases and Cell Morphology, Adhesion, and Motility

Rho, Rac, and Cdc42 are known to be key regulators of cytoskeletal changes in response to extracellular stimuli, and they also play a pivotal role in the regulation of cell migration and cell-cell and cell-substratum adhesion.53,54 More specific functions have been described for the 3 main GTPases in different cell types. Rho is involved in the formation of actin stress fibers and focal adhesions, membrane ruffling, and cell aggregation and motility.55–58 Rac regulates membrane ruffling, lamellipodium formation, actin polymerization, and cadherin-mediated cell-cell adhesion,59 whereas Cdc42 is implicated in filopodium formation,60 microtubule-dependent cell polarization and migration, and more recently, nucleus movement and positioning of the microtubule-organizing center (MTOC).61,62

In ECs, Rho GTPases have been implicated in a number of processes. Rho mediates stress fiber formation in response to extracellular stimuli such as thrombin, histamine,63 TNF-α,11 and shear stress,64 leading to actomyosin-mediated cell contraction, alterations in intercellular junctions, and increased endothelial permeability. Rho is also required for leukocyte adhesion to ECs, for clustering of ICAM-1 and VCAM-1, and formation of “docking” structures around adherent leukocytes.24,65 In addition, Rho, Rac, and Cdc42 are involved in shear stress-induced actin and microtubule cytoskeletal reorganization and cell polarization.56–59 Rac also regulates EC adhesion and migration on laminin-870 and the formation of new adhesion sites at the leading edge of migrating ECs by promoting the recruitment of αvβ3 integrins to that area.71

Rho GTPases and Gene Expression

Rho GTPases are known to modulate the activity of several transcription factors, leading to changes in gene expression. Rho GTPases are major regulators of actin dynamics, and some transcription factors, including serum response factor (SRF) and nuclear factor-kB (NF-kB), are sensitive to changes in the actin cytoskeleton.72,73 SRF transcriptional activity is induced by constitutively active forms of RhoA, Rac1, and Cdc42.74 This transcription factor recognizes specific DNA sequences called serum response elements located in the promoters of a variety of early-response genes, such as c-fos.75 c-Fos in turn is a component of the activator-protein-1 (AP-1) transcription factor, which contributes to the expression of leukocyte-binding endothelial receptors.76–78 Another component of AP-1, c-Jun, is regulated by RhoA in an actin polymerization-independent manner via ROCK-mediated JNK activation.79 RhoA, Rac, and Cdc42 have all been reported to activate NF-kB.80,81 Proinflammatory stimuli such as TNF-α, LPS, and IL-1 trigger the phosphorylation and subsequent polyubiquitination and proteosomal degradation of IκB, the inhibitor of NF-κB,82 allowing NF-κB to migrate to the nucleus where it interacts with specific sequences in promoters of genes, including ICAM-1, VCAM-1, and E-selectin in ECs.83,84

Rac can also regulate gene expression via NADPH oxidase activation and subsequent reactive oxygen species (ROS) generation.85 Originally, Rac was identified as an activator of the NADPH oxidase in phagocytic cells, but now it is known to regulate NADPH oxidases in other cell types as well, including ECs.86–88 In contrast, Cdc42 acts antagonistically to Rac and inhibits NADPH oxidase.89 ROS have multiple effects on cells, including activation of transcription factors such as AP-1 and NF-κB.90,91

Effects of Rho GTPases on the Expression and Function of Endothelial Receptors

E-Selectin and P-Selectin

As described above, the first step in leukocyte transmigration is the reduction in cell speed mediated by the interaction between selectins and cognate receptors such as PSGL-1 on the leukocyte surface, which is a prerequisite for subsequent firm adhesion.92 E-selectin is expressed in ECs in response to proinflamatory stimuli, such as bacterial endotoxin, IL-1β, or TNFα, through a mechanism involving NF-κB and JNK/p38 mitogen-activated protein kinase (p38MAPK) pathways.84,93 Rho GTPases are involved in TNF-α-induced E-selectin expression, as dominant-negative forms of RhoA, RhoB, and Rac1 inhibited this process.94 (Figure 1). Moreover, constitutively active Rac and Cdc42 augment E-selectin promoter activity in TNF-α–activated ECs.95 RhoA also regulates the localization of E-selectin. E-selectin on the endothelial surface clusters around adherent monocytes, and this clustering is inhibited by C3 transferase.65

P-selectin is regulated by proinflamatory stimuli in a different way. This protein is stored in ECs in Weibel-Palade Bodies (WPB), intracellular vesicles that fuse with the plasma membrane in response to a number of stimuli, including thrombin and histamine.97–99 These stimuli activate RhoA in ECs,100 but whether Rho proteins directly regulate P-selectin translocation to the EC surface is not known. RhoA has, however, been implicated in the clathrin-mediated internalization of P-selectin.101 In some circumstances, Rac1 is activated in ECs after thrombin stimulation.102 Rac activation correlates not only with WPB release but also with an increase in the production of ROS due to NADPH oxidase activation (Figure 1). WPB release is inhibited by dominant-negative Rac1 as well as by antioxidants, suggesting that P-selectin translocation to the EC surface is regulated by Rac1 through a ROS-dependent mechanism.102

Cross-linking of adhesion molecules with specific antibodies is a common technique used to study the effect of signaling through each receptor independently, and this approach has identified a number of downstream targets of leukocyte-binding receptors in EC. E- and P-selectin clustering increases cytosolic free calcium22,33 and induces changes in cell morphology32 and F-actin distribution.33
cross-linking also promotes its association with the EC cytoskeleton via its intracellular tail, localization in caveolin-containing lipid rafts, and its interaction with and activation of phospholipase Cγ. In addition, E-selectin cross-linking triggers activation of the MAPKs Erk1/2 and expression of the early-response gene c-fos.

The involvement of Rho GTPases in the processes triggered by E- and P-selectin engagement has not been explored, although a number of results suggest they could play a role. E-selectin localization to caveolin-rich lipid rafts may be important for its downstream effects. Rho proteins have been reported to localize to lipid rafts in different cell lines, and Rho and Rac affect Erk1/2 activation and SRF-mediated c-fos expression. Further studies regarding the role of Rho GTPases in E-/P-selectin signaling and whether this signaling occurs specifically in lipid rafts will clarify these points.

**Vascular Cell Adhesion Molecule-1**

VCAM-1 (CD106) is a molecule of particular interest in cardiovascular disorders, as its expression is induced early in nascent atheroma plaques. This member of the Ig superfamily of proteins binds to monocytes and T lymphocytes, both of which are found in nascent atheroma plaques, through its interaction with the integrin VLA-4/α4β1. VCAM-1 expression is induced by proinflammatory stimuli including TNF-α and IL-1β and is mediated, at least in part, by NF-κB. Nucleotides released under vascular stress conditions also induce VCAM-1 expression via activation of the P2Y2 receptor. Both Rho and Rac have been implicated in regulating VCAM-1 expression (Figure 2). Dominant negative RhoA inhibited UTP-induced VCAM-1 expression in coronary artery ECs. Under these conditions, an increase in Rac and Cdc42 activity was also observed, but Rac and...
Cdc42 were not directly involved in VCAM-1 expression. Dominant negative Rac1 inhibits VCAM-1 expression induced either by TNF-α via NF-κB or hypoxia/reoxygenation (H/RO) via ROS production.

In contrast to the effect of dominant–negative RhoA and Rac, statin pretreatment enhances VCAM-1 expression in TNF-α– or LPS-activated ECs. This enhancement is reversed by addition of geranylgeranyl-pyrophosphate. This suggests that a geranylgeranylated protein normally represses VCAM-1 expression. There are many geranylgeranylated proteins in addition to Rac and RhoA, and thus it will be important to test the specific effect of individual Rho GTPases on VCAM-1 expression in response to these stimuli.

In addition to binding leukocytes, VCAM-1 engagement contributes to leukocyte TEM by stimulating gap formation between cells in the endothelial monolayer, which could facilitate TEM by the paracellular pathway. This gap formation is mediated by VCAM-1–induced Rho and Rac activation and requires Rac-mediated ROS generation (Figure 2). ROS are involved in the activation of EC-associated matrix metalloproteinases (MMPs) MMP2 and MMP9 and actin reorganization triggered by VCAM-1 cross-linking. It is not clear what the targets of these MMPs are, but they could be involved in the shedding of adhesion molecules in cell-cell junctions and/or of VCAM-1 itself, thereby contributing to paracellular TEM.

VCAM-1 clustering also increases the intracellular free calcium concentration and causes Rho- and Rac-induced F-actin redistribution, both of which are necessary for leukocyte TEM.

Little is known about the effects of VCAM-1 engagement on long-term responses in ECs like changes in gene expression. It is probable that genes with promoters that are under the control of transcription factors directly regulated by Rho and Rac, as well as those sensitive to variations in the cellular redox status, such as NF-κB, AP-1, and hypoxia inducible factor-1, would be modulated.

Intercellular Adhesion Molecule-1

The 5 ICAMs are members of the Ig supergene family and are receptors for the β2 family of integrins on leukocytes.

ICAM-1 is specifically involved in the regulation of leukocyte trafficking across the endothelial barrier. Mice deficient in ICAM-1 display impaired inflammatory and immune responses, and antisense oligonucleotides to ICAM-1 decrease leukocyte adhesion and inflammation in a rat model of inflammatory bowel disease.

ICAM-1 is normally present in low levels on ECs, but its expression is dramatically increased in response to proinflammatory stimuli, including TNF-α, IL-1β, interferon-γ, phorbol myristate acetate, thrombin, and shear stress.

ICAM-1 surface levels are also regulated by proteolytic cleavage. In ECs, this is induced by cytokines and can be caused by MMP9 or the leukocyte elastase. Soluble ICAM-1 may modulate leukocyte adhesion and migration by interacting with β2 integrins on the leukocyte surface, but whether ICAM-1 shedding affects ICAM-1 signaling through its intracellular tail remains unclear.

Facilitating leukocyte attachment to the endothelial surface is not the only function of ICAM-1. Engagement of ICAM-1
also induces signaling in ECs, which is important for TEM.

The 28-amino acid ICAM-1 intracellular tail is responsible for signal transduction and interacts with F-actin and the cytoskeleton-associated proteins ezrin and α-actinin. ICAM-1 engagement triggers Src tyrosine kinase activity and induces the tyrosine phosphorylation of cortactin, focal adhesion kinase, paxillin, and p130Cas as well as RhoA activation and the formation of actin stress fibers. ICAM-1 can also activate the MAPKs Erk1/2 and/or JNK depending on the experimental system. Furthermore, the ICAM-1 cytoplasmic tail plays an important role in T-cell TEM via Rho GTPase activation and preferentially contributes to transcellular TEM. ICAM-1 clustering mediated either by leukocyte adhesion or antibody cross-linking induces ICAM-1 association to lipid rafts. Because RhoA and Src localize in part to lipid rafts, it is likely that ICAM-1 signaling in lipid rafts contributes to TEM.

Several studies have investigated the effect of ICAM-1 engagement on EC gene expression. In human umbilical vein ECs, ICAM-1 cross-linking has been reported to lead specifically to AP-1 activation without affecting NF-κB activity and to increase VCAM-1 expression. ICAM-1 also increases the production of IL-8 and RANTES through the activation of Erk1/2 and induces expression of ICAM-1, c-fos, and rhoA. Whether Rho GTPases contribute to these gene expression changes has not been investigated, but given that RhoA can regulate ICAM-1 and VCAM-1 expression, it is likely that ICAM-1 signaling in lipid rafts contributes to TEM.

Junctional Endothelial Receptors

One of the main functions of the endothelium is to act as a barrier between the bloodstream and the underlying tissues. Proteins localized at the cell-cell contact areas therefore play a pivotal role in controlling endothelial permeability. In addition, some cell-cell junction proteins can interact with leukocytes, including PECAM-1, JAMs and CD99, which may not only act as structural components of cell-cell junctions but also initiate signaling inside ECs.

PECAM-1

PECAM-1, also known as CD31, is an Ig superfamily adhesion molecule present in ECs, platelets, monocytes, and neutrophils. In ECs, PECAM-1 is localized at cell-cell contact areas and recycles between a juxta-membrane compartment and the plasma membrane. It has been shown to be involved in the regulation of endothelial permeability, participating in homophilic interactions between adjacent cells, leukocyte TEM, and endothelial motility.

Indirect evidence suggests that Rho GTPases regulate PECAM-1 expression on the EC surface. Statins not only affect the total levels of PECAM-1 in different EC lines but also modulate PECAM-1 localization. Moreover, C3 transferase treatment mimics the effect of statins on PECAM-1 expression.

PECAM-1 contributes to signal transduction in ECs via its intracellular tail, which is phosphorylated on mechanical stimulation of ECs, forms complexes with β-catenin and SH2-containing protein phosphatase, and regulates β-catenin transcriptional activity. It also modulates the phosphorylation state of signal transducer and activator of transcription family of proteins. At the plasma membrane, PECAM-1 is localized in caveolin-positive lipid rafts, potentially allowing it to interact with Rho proteins. In fact, PECAM-1-null ECs exhibit a decrease in active RhoA-GTP levels that is responsible for the increased motility of these cells.

JAMs

JAMs are Ig superfamily proteins localized mainly to cell-cell junctions in epithelial cells and ECs. The 3 main members of this family, JAM-A, JAM-B, and JAM-C, have been reported to be expressed in ECs. They are implicated in endothelial migration and paracellular TEM of leukocytes. They are involved in the adhesion of leukocytes to ECs via integrins or other JAMs expressed at the leukocyte surface. JAM-A binds to LFA-1 in neutrophils and T cells, whereas JAM-B has been reported to interact with VLA-4 and to promote lymphocyte TEM, and JAM-C is proposed to mediate neutrophil transepithelial migration by interacting with the integrin Mac-1 (CD11b/CD18).

In epithelial cells, constitutively active forms of RhoA and Cdc42 or treatment with the Rho-activating Escherichia coli cytotoxic necrotizing factor-1 induces re-localization of JAM-A away from the tight junctions. Although there is no direct evidence that Rho GTPases affect JAM localization in ECs, it is likely they will as Rho and Rac are known to mediate disruption of endothelial junctions induced by thrombin and proinflammatory cytokines. Loss of JAMs from the cell-cell junctions would be expected to alter leukocyte paracellular TEM.

JAMs have a short cytoplasmic tail that can be phosphorylated by protein kinases, which may be important for their activation. This intracellular tail interacts with PSD-95/Discs large/20-1 (PDZ)-containing proteins found in cell-cell junctions such as ZO-1, cingulin, occludin, and the cell polarity protein PAR-3. Connections between members of the JAM family and the Rho GTPases have not been identified, but a link is suggested by the observations that JAMs associate with PAR-3 and that PAR-3 interacts via PAR-6 with Rac1/Cdc42. It will therefore be interesting to determine whether JAMs affect Rho GTPase localization or activity. Whether JAMs also modify the gene expression pattern in ECs is not yet known.

Future Prospects

In recent years, our understanding of the role of EC receptors in the regulation of leukocyte TEM has evolved to assign a more active role to the EC in this physiological process. ECs respond to receptor engagement by modifying their morphology and the permeability of the monolayer. This takes place via changes in the actin cytoskeleton, cell adhesion sites, activation of MMPs, and gene expression, and Rho GTPases play a key role in these responses. Perhaps least is known about gene expression changes, but the upregulation of
adhesion molecules and other proinflammatory mediators point to a positive feedback mechanism acting in ECs to enhance the inflammatory response. Broader studies of changes in gene expression profile at both RNA and protein levels, for example using microarray analysis, will undoubtedly provide us with a more comprehensive understanding of the role played by ECs and Rho GTPases in inflammation and leukocyte TEM.

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