Adherent neutrophils activate endothelial myosin light chain kinase: role in transendothelial migration

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Increasing vascular endothelial cell (EC) permeability and neutrophilic leukocyte (PMN) diapedesis through paracellular gaps are cardinal features of acute inflammation. Activation of the EC contractile apparatus is necessary and sufficient to increase vascular permeability in specific models of EC barrier dysfunction. However, it is unknown whether EC contraction with subsequent paracellular gap formation is required for PMN transendothelial migration in response to chemotactic factors. To test this possibility, we assessed migration of human PMNs across confluent bovine pulmonary arterial EC monolayers. Transendothelial PMN migration in the absence of a chemotactic gradient was minimal, whereas abluminal addition of leukotriene B₄ (LTB₄; 5 µM) resulted in significantly increased PMN migration. Reduction in EC myosin light chain kinase (MLCK) activity by EC monolayer pretreatment with specific MLCK inhibitors (KT-5926 or ML-7) or by increases in cAMP-protein kinase A activity (cholera toxin) significantly reduced PMN transmigration (30–70% inhibition). In contrast, pretreatment with the myosin-associated phosphatase inhibitor calyculin resulted in the accumulation of phosphorylated myosin light chains, EC contraction, and significantly enhanced PMN migration. Finally, the interaction of PMNs with ³²P-labeled EC monolayers was shown to directly increase EC myosin phosphorylation in a time-dependent fashion. Taken together, these results are consistent with the hypothesis that the phosphorylation status of EC myosin regulates PMN migration and further indicate that EC MLCK is activated by chemoattractant-stimulated PMNs. Neutrophil-dependent activation of the EC contractile apparatus with subsequent paracellular gap formation may be a key determinant of transendothelial PMN migration responses to chemotactic agents.

inflammation; cytoskeleton; paracellular gap formation; neutrophil diapedesis

The margination and migration of circulating neutrophilic leukocytes (PMNs) are critical components of the inflammatory process. Emigration of PMNs from the blood into the extravascular tissue is a multistep process, with leukocytes rolling along the postcapillary vascular wall with subsequent tethering to the endothelial cell (EC) surface. PMN migration through the vessel wall is regulated in a complex fashion by specific recognition molecules on PMN and EC plasmalemmal membranes (1, 10, 13). The consequences of the intimate cell-cell association between EC and adhering PMNs are poorly understood. However, this interaction likely exerts potentially important cross-cellular regulatory influences on signaling pathways on both the endothelium and responding PMNs. For example, Cui et al. (2) showed that PMN chemotactic migration across confluent endothelium, in the presence of a chemotactic gradient produced by leukotriene B₄, resulted in activation of EC phospholipase D. Neither direct EC challenge with LTB₄, nor simple physical contact of EC with PMNs in the absence of the chemotactic gradient was sufficient to trigger phospholipid D activation (2).

Increasing information regarding juxtacrine interactions between adherent or migrating PMNs and EC has raised the possibility that similar cross-cellular signaling pathways may be involved in the process of PMN migration into inflamed tissues. For example, EC responses to adherent PMNs may lead to a loss of vessel wall integrity and the formation of paracellular gaps, thereby facilitating PMN migration. Paracellular gap formation is now recognized as critical to enhanced vascular permeability to solutes and fluids (4, 6, 12, 18), which, like PMN diapedesis, is a key feature of the inflammatory process. The premise that PMNs may regulate EC paracellular gaps was first postulated by Huang et al. (9), who described increases in EC cytosolic Ca²⁺ in response to adherent PMNs. Similar increases in EC cytosolic Ca²⁺ are evoked by adherent tumor cells (16). The recognized role of Ca²⁺ in regulating the integrity of the EC monolayer (6, 7, 12, 18) opens the possibility that adherent, migrating PMNs may also regulate EC paracellular gap formation.

Although the biochemical and molecular processes that regulate the vascular paracellular space are incompletely understood, it has been proposed that EC monolayer integrity is maintained by a balance of barrier-disrupting contractile forces existing in equilibrium with tethering forces comprised of adherens junction and focal adhesion proteins (6, 12, 14). In previous work, our laboratory has shown that the phosphorylation status of EC myosin light chains (MLCs) is a key determinant of EC gap formation (4, 21). Our laboratory has cloned and characterized the critical involve-
mment of a novel high-molecular-weight EC MLC kinase (MLCK) isofrom in EC contraction, paracellular gap formation, and barrier dysfunction (3, 5). The activity of this contractile effector appears to be regulated by cytosolic Ca\(^{2+}\) as well as by the phosphorylation status of the kinase (4, 5). For example, cAMP-dependent protein kinase A (PKA)-mediated increases in EC MLCK phosphorylation reduce MLCK activity (5), thereby abolishing EC contraction and gap formation evoked by bioactive agents (4, 15). Furthermore, inhibition of myosin-associated type 1 serine-threonine (Ser/Thr) phosphatase activity produces significant EC contraction and paracellular gap formation because of the accumulation of phosphorylated myosin (21).

In this study, we hypothesized that the MLCK-dependent activation of the EC contractile apparatus regulates PMN diapedesis by allowing PMN to migrate between EC into inflamed tissues. Our data indicate that PMN-EC interaction in response to chemoattractant stimuli produces EC MLCK activation, thereby facilitating EC gap formation and transendothelial migration. These results suggest EC contractile proteins play an essential role in regulating the migration of leukocytes into sites of infection and inflammation.

**MATERIALS AND METHODS**

Reagents. Chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO). Transwell chambers (24 mm diameter, 3 µm pore size) for EC culture and PMN chemotaxis were obtained from Costar (Cambridge, MA). Bovine pulmonary arterial EC were from American Type Culture Collection (Rockville, MD).

Bovine pulmonary artery EC culture. EC were cultured as described previously (4, 15) under a humidified atmosphere of 95% air-5% CO\(_2\). After 16–20 passages in gelatin-coated T-75 cm\(^2\) flasks, the cells were trypsinized and seeded onto the 3-µm polycarbonate filters at the base of inserts of the added, and the Transwell plates were placed in a CO\(_2\) incubator at 37°C in a humidified atmosphere of 5% CO\(_2\)-95% air. At specific times after PMN contact, proteins were harvested by precipitation into ice-cold 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol. The cellular proteins present in TCA-treated pellets were washed three times with diethyl ether and resuspended in sample buffer containing 6.7 M urea and (in mM) 10 dithiothreitol, 20 Tris, 22 glycerol, and 270 sucrose, pH 9.0. MLC phosphorylation was analyzed as we have described by a modification of the urea PAGE separation (20 µg/lane) of the mono- and diphosphorylated forms of MLC, which migrate more rapidly than do unphosphorylated forms (4). Radioactive protein bands were detected by autoradiography at —70°C. Positions of both phosphorylated and unphosphorylated MLC were detected by immunoblotting with 1:1,000 MLC-specific antibody followed by staining with goat anti-rabbit-horseradish peroxidase system (4). EC MLCK phosphorylation was measured by densitometry of autoradiographs of nitrocellulose blots containing phosphorylated labeled MLC and normalized by protein loading.

**RESULTS**

Effect of EC MLCK inhibition on PMN migration. Transendothelial migration of human PMNs across confluent EC monolayers over a 2-h period in the absence of LTB\(_4\) was significantly less than migration after the addition of LTB\(_4\) (5 µM) to the abluminal media (Fig. 1). The increase in PMN transendothelial migration began as early as 5 min and plateaued between 60 and 120 min. In prior work, our laboratory has shown that the addition of equimolar amounts of LTB\(_4\) to both the upper and lower chambers fails to significantly augment PMN migration into the ablumi-
The endothelium is a key participant in multiple components of inflammatory processes. For example, tissue edema, a cardinal feature of the acute inflammatory response to chemotactic signals, is critical to PMN diapedesis.

DISCUSSION

Given its role as a semiselective diffusion barrier between the plasma and interstitial fluid, the vascular endothelium is a key participant in multiple components of inflammatory processes. For example, tissue edema, a cardinal feature of the acute inflammatory...
response, is the result of a complex but well-orchestrated series of biochemical events that are linked to paracellular gap formation evoked by permeability-inducing bioactive agents (6, 12). Evidence now exists that EC gap formation is controlled, at least in part, by actin and/or myosin interactions that are regulated via the phosphorylation of MLCs, an event catalyzed by Ca2+/calmodulin-dependent MLCK (4, 8, 11, 21). In recent reports we have characterized the involvement of this kinase in EC gap formation and permeability responses (4), with molecular cloning revealing the presence of a novel MLCK isoform in vascular endothelium (5).

In addition to increases in vascular permeability, the margination and subsequent migration of circulating PMNs through the endothelium into inflamed tissues are other critical components of the inflammatory process (1, 10, 13). After metabolic activation, PMNs mediate EC damage associated with the inflammatory response (17). However, in addition to causing direct vascular injury, PMNs may also disrupt vascular EC monolayer integrity by inducing responses in EC during the process of chemotactic migration (1, 2, 19, 22). In prior studies, our laboratory has demonstrated that PMN-EC interaction and subsequent transendothelial migration result in activation of EC phospholipase D (2), thereby generating important inflammatory mediators such as phosphatidic acid and diacylglycerol. The data reported in the present study indicate that the activity of EC MLCK, via its capacity to increase EC contraction and paracellular gap formation, is critical to the migration of chemoattractant-stimulated PMNs. Subsequent experiments performed with radiolabeled EC demonstrated that adherent PMNs can directly increase EC MLC phosphorylation, with maximal effect elicited in the presence of a chemoattractant gradient. Because increases in EC MLC phosphorylation were not observed in EC exposed to LTB4 in the absence of PMNs, our results are consistent with our hypothesis that PMN-EC interaction, particularly in the presence of a chemotactic stimulus, is essential and sufficient to activate EC MLCK.

Our experiments demonstrate that direct EC MLCK inhibition via pharmacological inhibitors (KT-5926 and ML-7) or via posttranslational modification (cholera toxin) decreased PMN transmigration in response to a chemoattractant stimulus. Increasing cAMP-dependent PKA activity with cholera toxin (7, 15) attenuates MLCK activation (5); abolishes thrombin-induced MLC phosphorylation, EC gap formation, and permeability (4); and reverses established barrier dysfunction subsequent to thrombin activation (15). Although the exact PKA substrates involved in regulating PMN diapedesis are unknown, cholera toxin does not prevent adhesion of PMNs to EC monolayers (20). Thus it is unlikely that the protective effects of cAMP on PMN-mediated EC barrier dysfunction (20) or on PMN migration (Fig. 2) can be ascribed to a reduction in PMN adhesion. We speculate that the effect of cholera toxin on PMN migration and macromolecule permeability occurs, at least in part, via its capacity to reduce MLCK phosphorylation (5), MLC phosphorylation (4), and subsequent paracellular gap formation (15).

In prior work our laboratory has shown that, in addition to MLCK activity, the phosphorylation status of EC MLC is determined by specific type 1 Ser/Thr myosin-associated phosphatase activities (21). Inhibition of this phosphatase activity with the protein phosphatase inhibitor calyculin A produced dose-dependent enhancement of both EC MLC phosphorylation and EC permeability (21). In the present study, compared with vehicle-pretreated controls, PMN transmigration through EC monolayers pretreated with calyculin was significantly increased in the presence of the chemoattractant agent LTB4. Although we have not excluded a potential effect of calyculin on target proteins other than MLC, these data are consistent with our hypothesis that PMN migration into tissues occurs through EC intercellular gaps linked to the phosphorylation of MLCs.
Huang et al. (9) noted that the rise in EC Ca$^{2+}$ and/or receptor complexes that remain undefined (22). With a role for PMN-stimulated EC intracellular Ca$^{2+}$ adhesion but was essential for EC gap formation and cytosolic Ca$^{2+}$ decreases in EC monolayer electrical resistance, and though a prompt rise in Ca$^{2+}$ mobilization in subsequent EC MLCK activation. Al-
mediated increases in EC Ca$^{2+}$ chemotactant-stimulated PMNs produce EC MLCK tissue inflammation. The exact mechanism by which the migration of leukocytes from the blood into sites of tissue inflammation. The exact mechanism by which chemotactant-stimulated PMNs produce EC MLCK activation is not known but likely involves PMN-mediated increases in EC Ca$^{2+}$ (9) through ligand and/or receptor complexes that remain undefined (22). Huang et al. (9) noted that the rise in EC Ca$^{2+}$ evoked by PMN-EC interaction was not required for PMN adhesion but was essential for EC gap formation and PMN migration through EC monolayers, consistent with a role for PMN-stimulated EC intracellular Ca$^{2+}$ mobilization in subsequent EC MLCK activation. Although a prompt rise in Ca$^{2+}$ precedes maximal thrombin-mediated EC gap formation, MLC phosphorylation, decreases in EC monolayer electrical resistance, and increases in EC monolayer permeability (4), a rise in cytosolic Ca$^{2+}$ alone is insufficient to activate EC MLCK (4, 7). Furthermore, characterization of additional PMN-elicted EC signaling pathways that modulate EC MLCK activity and the EC contractile apparatus awaits further study.

In summary, our data indicate that the MLCK-driven EC contractile apparatus is an active participant in transendothelial PMN migration in response to chemotactic agents. These data support the concept of cross-cellular activation of specific EC signaling pathways by adherent or migrating PMNs. Our results suggest a model in which chemotactant-stimulated PMNs induce phosphorylation of MLC (and possibly other endothelial cytoskeletal proteins), increasing EC contractility and intercellular gap formation, thereby regulating the migration of leukocytes from the blood into sites of tissue inflammation. The exact mechanism by which chemotactant-stimulated PMNs produce EC MLCK activation is not known but likely involves PMN-mediated increases in EC Ca$^{2+}$ (9) through ligand and/or receptor complexes that remain undefined (22).

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REFERENCES
18. Schnittler, H. J., A. Wilke, T. Gress, P. N. Suttorp, and D. Drenckhahn. Role of actin and myosin in the control of paracel-