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Endothelial Myosin Light Chain Kinase Regulates Neutrophil Migration Across Human Umbilical Vein Endothelial Cell Monolayer

Hajime Saito,* Yoshihiro Minamiya,‡ Michihiko Kitamura,* Satoshi Saito,* Katsuhiko Enomoto,† Kunihiko Terada,‡ and Jun-ichi Ogawa*

Although extravasation of neutrophils is a critical step in acute inflammation, the role of the endothelial cytoskeleton in neutrophil transmigration has not been fully investigated. We used an in vitro model of neutrophil transmigration across a monolayer of HUVEC cultured on amniotic membrane. Human neutrophils were allowed to migrate across the HUVEC monolayer in response to a gradient leukotriene B4 and then the number of migrated neutrophils were counted microscopically. We also followed endothelial F-actin and myosin filament formation using rhodamine-phalloidin and anti-myosin Ab staining. Myosin light chain (MLC) phosphorylation in endothelial cells was determined by immunoprecipitation of 32P-labeled HUVEC with anti-myosin polyclonal Ab. Normally, neutrophil migration induced F-actin formation, myosin filament formation, and MLC phosphorylation in HUVEC. When HUVEC was pretreated with the myosin light chain kinase (MLCK) inhibitor, ML-9, neutrophil migration was diminished and F-actin formation, myosin filament formation, and MLC phosphorylation were inhibited. Pretreatments of HUVEC with the intracellular calcium ion chelator, bis-(O-aminophenoxy)ethane-N,N',N''-tetraacetic acid acetoxymethyl ester (BAPTA/AM), and the calmodulin antagonist, trifluoperazine, had similar effects. These results indicate that a calcium/calmodulin-dependent MLCK in endothelial cells regulates neutrophil transendothelial migration.


A critical step in acute inflammation is migration of the circulating neutrophil from the vascular compartment through a gap between adjacent endothelial cells into surrounding tissue (1–3). Substantial evidence has accumulated demonstrating that adhesion molecules, such as β2 integrin (4–7), ICAM-1 (6–8), and platelet endothelial cell adhesion molecule-1 (PECAM-1)9 (9–11), are required for initiation of neutrophil transendothelial migration. Mechanisms involved after neutrophil adhesion have received less attention. In particular, signaling mechanisms triggered within endothelial cells during neutrophil transmigration are not well understood. Huang et al. have provided data indicating that cytosolic calcium-dependent endothelial signaling results in creation of gaps at junctions between adjacent endothelial cells through which neutrophils are able to pass (12). There are two kinds of endothelial cell junctions (ECJ) described as tight junctions and adherence junctions (13, 14), both displaying discontinuities at the tricellular corner where the majority of neutrophils migrate across (14).

Phosphorylation of myosin II light chain (MLC) by a calcium/calmodulin-dependent kinase is considered to be an essential step in contraction of smooth muscle cells (15–18) and other cells (19–21) through interaction of actin and myosin (22, 23) and formation of myosin II filaments (24–27). There have also been reports that the actin filament binds directly to the adherence junction-associated protein, α-catenin (28, 29), and that α-spectrin, which is cross-linked to the actin filament, binds to the tight junction associated-protein, ZO-1 (30). Histamine and thrombin-induced phosphorylation of MLC have been shown to initiate endothelial cell retraction (31–35) and to result in disassembly of the adherence junction complex (36). Taken together, these findings support a hypothesis that phosphorylation of MLC induces opening gaps between endothelial cells associated with tricellular corners and with ECJ.

In the present study we examine the hypothesis that MLCK in endothelial cells plays an active role in transendothelial migration of neutrophils. To assess MLC phosphorylation during neutrophil transmigration, we used an in vitro model consisting of a monolayer of HUVEC cultured on amniotic membrane (37). We also investigated the effect of inhibition of MLCK on migration of neutrophils across a HUVEC monolayer. Our results are in accord with a calcium/calmodulin-dependent MLCK acting to regulate transendothelial neutrophil migration.

Materials and Methods

Abs and reagents

Anti-human platelet myosin II rabbit polyclonal Abs (32) (anti-M II pAb) was kindly provided by Dr. Robert Wyssolmerski (St. Louis University, St. Louis, MO). The anti-chicken myosin light chain mouse mAb MY-21, which cross-reacts with human MLC, was purchased from Sigma (St.

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Louis, MO). L-leskotriene B4 (LTB4) was kindly provided by Ono Pharmaceutical (Osaka, Japan). Rhodamine-phalloidin and bis-(O-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAIP/ AM) were purchased from Molecular Probes (Eugene, OR). Trifluoperazine and ML-9 were purchased from Calbiochem (La Jolla, CA) and Seikagaku Kogyo (Tokyo, Japan), respectively.

**Assay of transendothelial migration of neutrophils**

**HUVEC culture.** HUVEC were harvested by perfusion of umbilical vein with 0.25% trypsin (Life Technologies, Grand Island, NY) according to the modified method of Jaffe et al. (38). Cell preparations were transferred to 60-mm plastic tissue culture dishes coated with type I collagen (Sigma), and HUVEC were grown in M199 medium (Life Technologies) supplemented with 20% FCS, 100 U/ml penicillin G, and 100 µg/ml streptomycin while being maintained at 37°C in a humidified 5% CO2, 95% air atmosphere for 1 min and incubated for 60 min with 2% formaldehyde in PBS containing 0.1 M N-propylgallate and 0.1% BSA (final Ab concentration: 0.75 mg/ml). After rinsing with PBS containing 0.1% BSA, the membrane was incubated at room temperature for 1 h with secondary Ab, anti-rabbit IgG PAb conjugated with rhodamine. After washing with PBS, several drops of 90% glyceral/10% PBS containing 0.1 M N-propylgallate were added and the membrane was covered with a coverslip. Finally, the membrane was examined using a confocal laser scanning microscope system (LSM 410, Zeiss, Oberkochen, Germany) coupled to a Axioverd 135 fluorescence microscope (Zeiss).

**Myosin light chain phosphorylation**

To analyze myosin phosphorylation, myosin was immunoprecipitated as described by Wysolmerski and Lagunoff (35, 41) with minor modifications. A confluent monolayer of HUVEC on an amniotic culture ring (diameter 90 mm) was labeled with [32P]orthophosphoric acid (DuPont-NEN, Boston, MA) as follows. HUVEC were washed twice with low phosphate medium (DMEM, Life Technologies) and then incubated for 3 h at 37°C (humidified 5% CO2, 95% air) with 75 µCi/ml of [32P]orthophosphoric acid in the same. After washing HUVEC with PBS to remove excess free [32P]orthophosphoric acid, a neutrophil transendothelial migration assay was performed. Briefly, 2.0 × 106 neutrophils were added to the upper compartment of [32P]-labeled HUVEC on an amniotic culture ring and LTB4 was added to the lower compartment to a concentration of 10−7 M. After being incubated for 10, 30, or 60 min, HUVEC on the amniotic culture ring were washed three times with cold PBS, lysed with 600 µl of the lysis buffer (25 mM Tris-HCl, pH 7.9, 250 mM NaCl, 100 mM Na2PO4, 75 mM NaF, 0.5% sodium deoxycholate, 1% Nonident P-40, 5 mM EDTA, 5 mM EGTA, 0.2 mM PMSF, and 10 µg/ml leupeptin), incubated on ice for 30 min and scraped with a rubber policeman. After rinsing the amniotic culture membrane with 200 µl of the lysis buffer, the soluble cell extract was centrifuged at 132,000 × g for 10 min and the supernatant was collected. The pellet was extracted again by incubation for 20 min with 200 µl of the lysis buffer containing 600 mM NaCl. The insoluble materials were pelleted at 132,000 × g. The supernatant was diluted with an equal volume of the lysis buffer without NaCl and then combined with the initial sample. To avoid nonspecific binding, the sample was preincubated for 30 min at 4°C with 100 µl of 20% skim milk (pH 7.4) and 20 µl of 50% protein NG-Sepharose, centrifuged for 10 min at 15,000 × g, and the supernatant was removed. The supernatant was incubated overnight at 4°C with 20 µl of anti-M II PAb (12.5 mg/ml). The next day the sample was incubated for 2 h at 4°C with 20 µl of 50% protein NG-Sepharose and the immune complexes bound to affinity media were collected by centrifugation for 5 min at 12,000 × g. The pellets were washed first in 1 ml of lysis buffer, then washed once with a 1:1 dilution of lysis buffer/PBS, twice with PBS and, finally, once with a 1:1 dilution of PBS/distilled water. Then pellets were boiled in Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, ...
60 nM Tris-HCl, pH 6.8, and 0.001% bromphenol blue) and subjected to polyacrylamide gel electrophoresis (12–15%). The gel was stained with Coomassie blue, dried using a gel drier, and exposed to Fuji RX-1 x-ray film. The film was developed with a Konica automatic processor SRX-101.

Inhibitory experiments

To determine the effect of chelation of intracellular calcium ion ([Ca$^{2+}$]) and of inhibition of calmodulin and MLCK on the endothelial cytoskeleton and on transmigration of neutrophils, a series of experiments were conducted. HUVEC on an amniotic culture ring or a polycarbonate membrane were preincubated at 37°C for 30 min with the [Ca$^{2+}$]_i chelator, BAPTA/AM (50, 100, 200 μM), with the calmodulin inhibitor, trifluoperazine (10, 50, and 100 μM), or with the MLCK inhibitor, ML-9 (5, 50, and 300 μM). After washing three times with PBS, assay of neutrophil transendothelial migration, staining of actin and myosin II, and assessment of MLC phosphorylation were performed as before.

Statistics

Values are expressed as the mean ± SD. The significance of any difference between groups was assessed by one-way analysis of variance with the Scheffe’s multiple comparison test. Differences between groups were considered significant for any p < 0.05.

Results

Neutrophil transendothelial migration assay

Figure 1 shows the experimental design of the neutrophil migration assay. The number of neutrophils migrating across the HUVEC monolayer toward LTB$_4$ was quantified by actual counting assay. The number of neutrophils migrating across the HUVEC monolayer was observed between 15 and 30 min. Neutrophils beneath the HUVEC monolayer were defined as migrated neutrophils and were quantified as the number of neutrophils/mm$^2$.

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Table I. Effect of inhibitors on neutrophil adherence to HUVEC

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Dose (μM)</th>
<th>No.</th>
<th>Adherent Neutrophils (/mm²)</th>
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</thead>
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<tr>
<td>BAPTA/AM</td>
<td>0</td>
<td>4</td>
<td>27.0 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>33.0 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>29.2 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5</td>
<td>25.8 ± 6.6</td>
</tr>
<tr>
<td>Trifluoperazine</td>
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<td>3</td>
<td>26.0 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>30.0 ± 4.7</td>
</tr>
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<td></td>
<td>50</td>
<td>5</td>
<td>24.6 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>31.2 ± 4.9</td>
</tr>
<tr>
<td>ML-9</td>
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<td>4</td>
<td>28.0 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
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<td>29.6 ± 5.9</td>
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<td>33.2 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>5</td>
<td>31.2 ± 6.3</td>
</tr>
</tbody>
</table>

* After HUVEC were pretreated with inhibitors (BAPTA/AM, trifluoperazine, and ML-9) as before, neutrophils and LTB$_4$ were added to the upper and lower compartments of the HUVEC amniotic culture ring, respectively. Following an incubation at 37°C for 30 min, HUVEC were washed with PBS three times. The number of adherent neutrophils was then counted using a microscope. There was no significant difference in the number of neutrophils adhering to HUVEC pretreated with inhibitors when compared with untreated HUVEC.
FIGURE 4. Immunofluorescence labeling of endothelial F-actin and myosin II during neutrophil transendothelial migration. Time course of redistribution of F-actin (a–f) and myosin II (g–l) is shown. Neutrophils (2.0 × 10^6) were layered above a HUVEC monolayer on a polycarbonate membrane insert and medium in the lower compartment of the well was made 10^{-7} M with respect to LTB₄. Then, the 24-well plate was incubated at 37°C for different periods of time (10, 30, and 60 min). F-actin was visualized by rhodamine-phalloidin staining. Myosin II was visualized by indirect immunostaining with
F-actin and myosin II reorganization during neutrophil transendothelial migration

To assess actin and myosin II reorganization during neutrophil transendothelial migration, HUVEC were stained with rhodamine-phalloidin and anti-M II pAb, respectively. In the absence of neutrophils and LTB₄, a rim of F-actin staining was present at the margins of cells, with a few randomly disoriented stress fibers within the cytoplasm (a). Myosin II was diffusely localized within the cytoplasm and exhibited no obvious organization (b–f). These patterns were identical to those observed in controls (Fig. 4, a, g). Therefore, pretreatment with the inhibitors prevented the formation of actin and myosin II filaments in endothelial cells, changes induced by neutrophil transmigration (Fig. 4, b–d and h–j).

FIGURE 5. Effects of inhibitors on endothelial F-actin and myosin II redistribution. A monolayer of HUVEC on a polycarbonate membrane insert was pretreated with the [Ca²⁺]ᵢ chelator, BAPTA/AM (200 µM), the calmodulin inhibitor, trifluoperazine (100 µM), or the MLCK inhibitor, ML-9 (50 µM). After washing with PBS three times, neutrophils (2.0 × 10⁶) were layered above the HUVEC monolayer, and medium in the lower compartment of the well was made 10⁻⁷ M with respect to LTB₄. Next, the 24-well plate was incubated for 30 min at 37°C. Then the polycarbonate membrane insert was fixed and stained with rhodamine-phalloidin (a–c) and anti-M II pAb followed by rhodamine-labeled anti-rabbit IgG Ab (d–f). Panels a and d, b and e, and, c and f depict HUVEC treated with BAPTA/AM, trifluoperazine, and ML-9, respectively. The images were observed using confocal laser scanning microscopy. A rim of F-actin staining was present at cell margins with a few randomly disoriented stress fibers within the cytoplasm (a–c). Myosin II was diffusely localized within the cytoplasm and exhibited no obvious organization (d–f). These data are identical to those observed in controls (Fig. 4, a, g). Therefore, pretreatment with the inhibitors prevented the formation of actin and myosin II filaments in endothelial cells, changes induced by neutrophil transmigration (Fig. 4, b–d and h–j).
agents also prevented changes in HUVEC F-actin and myosin II filament formation. HUVEC monolayers were pretreated with 200 μM of BAPTA/AM, 100 μM of trifluoperazine, or 50 μM of ML-9, respectively, and F-actin and myosin II staining were repeated. A rim of F-actin staining was still present at cell margins with a few randomly disoriented stress fibers within the cytoplasm of endothelial cell (Fig. 5a–c), and myosin II staining remained diffusely localized within the cytoplasm of cells and exhibited no obvious organized pattern (Fig. 5, d–f). Therefore, pretreatment with all three inhibitors almost completely inhibited endothelial MLC phosphorylation (b, lane 1, BAPTA/AM; lane 2, trifluoperazine; lane 3, ML-9; lane 4, positive control at 60 min).

Endothelial MLC phosphorylation during neutrophil transendothelial migration

To further investigate the role of endothelial MLCK, we studied MLC phosphorylation during neutrophil transmigration. Endothelial MLC was progressively phosphorylated in a time-dependent manner reaching near maximal levels at 30 min (Fig. 6a). We also determined the effects of inhibitors on this process. BAPTA/AM, trifluoperazine, and ML-9 all almost completely inhibited endothelial MLC phosphorylation (Fig. 6b).

Discussion

In this study we used HUVEC cultured on anamnestic membranes to investigate the role of endothelial MLCK during neutrophil transmigration. Isolated human neutrophils were allowed to migrate across the HUVEC monolayer in response to LTB4. Using this model system, we demonstrated that endothelial MLC phosphorylation, endothelial actin, and myosin II filament formation all occurred during neutrophil migration across HUVEC monolayer. We also demonstrated that pretreatment of HUVEC with the MLCK-specific inhibitor, ML-9, inhibited neutrophil migration across HUVEC monolayer as well as MLC phosphorylation and filament formation in endothelial cells.

The most critical finding in this investigation was that ML-9 inhibited neutrophil migration across a HUVEC monolayer. ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-IH-hexahydro-1,4-diazepine, binds at or near the ATP-binding site of the active center, resulting in inhibition of the catalytic activity of MLCK (42). Although, ML-9 inhibits the catalytic activities of the other enzymes, the inhibition constant (K_i) values are at least an order of magnitude higher than for MLCK (42). For example, the K_i values of cAMP-dependent protein kinase, protein kinase C, and Ca^2+ phosphodiesterase are 32 μM, 54 μM, and 50 μM, respectively. The K_i value of ML-9 for MLCK was 3.8 μM. In the present study, incubation with 5 μM of ML-9 inhibited 50% of neutrophil transmigration (Fig. 3) and we feel that inhibition at this concentration would be specific for MLCK. The IC_{50} (concentration producing a 50% inhibition) in a model of smooth muscle contraction by high K^+ was 17.5 ± 0.4 μM. In our experiments, we used 50 μM of ML-9 and this concentration almost completely inhibited the effects of neutrophil migration on mechanisms within the endothelial cells, including actin and myosin II filament formation and MLC phosphorylation (Figs. 3, 5, and 6). Although this value was a three times higher concentration of the IC_{50}, it still did not reach to IC_{50} for other kinases estimated from K_i values. Therefore, we concluded that endothelial MLCK regulates neutrophil migration across a HUVEC monolayer.

It has previously been reported that MLCK in the endothelial cell is [Ca^{2+}]/calmodulin dependent (41). Therefore, to determine effects of chelation of [Ca^{2+}], and calmodulin inhibition in endothelial cells, we repeated experiments after pretreating HUVEC with BAPTA/AM or trifluoperazine. Pretreatment with inhibitors inhibited neutrophil migration across the HUVEC monolayer as well as endothelial actin and myosin II filament formation, and MLC phosphorylation. These results indicate that a [Ca^{2+}]/calmodulin-dependent MLCK in endothelial cells regulates neutrophil transendothelial migration.

It has been well described that phosphorylation of MLC induced actin polymerization, endothelial cell centripetal retraction, and a subsequent increase in the endothelial permeability (31–35). We speculate that the same mechanism regulates neutrophil trafficking across the endothelial barrier. The fact that actin and myosin II filament formation, which we observed in endothelial cells during neutrophil migration, were similar to cytoskeletal changes observed by other investigators in thrombin-stimulated endothelial cells (32) supports our speculation. On the other hand, other mechanisms capable of regulating MLC phosphorylation, such as protein kinase C (43), cAMP-dependent protein kinase A (43), and myosin phosphatase inhibition (44) by Rho-kinase (45–47) were recently reported. Thus, it is necessary to further investigate mechanisms contributing to endothelial MLC phosphorylation during neutrophil transmigration and to examine the relationship between these enzymes and MLCK.

The present study and previous studies provide evidence that endothelial cell-dependent mechanisms regulate neutrophil transmigration. Huang et al. demonstrated that cytosolic-free calcium in the endothelial cell regulates neutrophil transendothelial migration (12). This study suggests that some cytosolic calcium-dependent endothelial signaling mechanisms open a gap between adjacent endothelial cells through which neutrophils can pass. Alloport et al. (48) reported that neutrophil endothelial adhesion triggers the disruption and degradation of the VE-cadherin complex at the adherence junction. These endothelial cell-dependent changes then regulate neutrophil transendothelial migration. On the other hand, Burns et al. recently demonstrated that 75% of neutrophils migrate across at locations where the borders of these endothelial cells intersect, at tricellular corners. Here, tight junctions and adherence junctions have discontinuities (14). The relationship between tricellular corners and the cytoskeleton of endothelial cells has not been investigated. The present study demonstrated that endothelial...
cytoskeleton regulates neutrophil transendothelial migration. Taken together, we speculate that tricellular corners may also be regulated by the endothelial cytoskeleton.

We have demonstrated an active role for endothelial [Ca$^{2+}$]/calmodulin-dependent MLCK in neutrophil transendothelial migration. However, “the switch” that opens gaps between adjacent endothelial cells at tight junctions, adherence junctions, and tricellular corners remains unclear. Neutralization with Abs to $\beta_2$ integrin on the neutrophil (4–7), ICAM-1 on the endothelial cell (6–8), PECAM-1 on the endothelial cell, and the neutrophil (9–11) or CD47 on the neutrophil (49) all inhibited neutrophil transendothelial migration. Therefore, ICAM-1, PECAM-1, and the unknown ligand for CD47 on the endothelial cell are candidates for being “the switch” on the endothelial cell required to open a gap between adjacent endothelial cells. We speculate that neutrophils may stimulate “the switch” and that “the switch” may then release a signal to activate [Ca$^{2+}$]/calmodulin-dependent MLCK. In turn, MLCK catalyzes myosin II filament formation (24–27) and myosin-actin interaction (22, 23), and induces actin polymerization. It was reported that actin filament binds directly to the adherence junction-associated protein, $\alpha$-catenin (28, 29), and that the tight junction-associated protein, ZO-1, binds directly to a cross-linking protein of the actin filament, $\alpha$-spectrin (30). Therefore, actin polymerization may directly open these junctions. In fact, Rabiet et al. (36) demonstrated that thombin, which phosphorylates MLCK (31–35), induced disassembly of the adherence junction complex (36). The relationship between the tricellular corner and the cytoskeleton of endothelial cells has not been investigated. It is possible that the tricellular corner may be regulated by endothelial signal transduction related to MLCK. Although there are many signaling steps to be investigated, we feel we have clarified some parts of the signal transduction mechanism within the endothelial cell that is induced during neutrophil transendothelial migration.

In summary, we have demonstrated that endothelial MLC was phosphorylated during neutrophil migration across a HUVEC monolayer. We have also shown that pretreatment of HUVEC with the MLCK-specific inhibitor, ML-9, inhibited neutrophil migration across a HUVEC monolayer toward LTBI. ML-9 pretreatment of HUVEC also prevented endothelial actin and myosin II filament formation and endothelial MLC phosphorylation. Furthermore, pretreatments of HUVEC with a [Ca$^{2+}$]/chelator and a calmodulin antagonist caused similar inhibitions. These results indicate that an endothelial [Ca$^{2+}$]/calmodulin-dependent MLCK regulates neutrophil transendothelial migration.

Acknowledgments

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References