The extracellular matrix protein mindin serves as an integrin ligand and is critical for inflammatory cell recruitment

Wei Jia, Hong Li, and You-Wen He

Leukocyte recruitment to inflammation sites depends on interactions between integrins and extracellular matrix (ECM). In this report we show that mice lacking the ECM protein mindin exhibit severely impaired recruitment of neutrophils and macrophages in 4 different inflammation models. Furthermore, neutrophils directly bind to immobilized mindin, and mindin matrix mediates neutrophil migration in vitro. The adhesion of neutrophils to mindin is blocked by anti-integrin αM, anti-integrin αδ, and anti-integrin β2 antibodies. We also show that HEK-293 cells transfected with cDNA encoding these integrins exhibit enhanced binding to immobilized mindin matrix and the increased binding can be blocked by anti-integrin antibodies. Our results suggest that mindin serves as a novel ligand for integrins and mindin-integrin interactions are critical for inflammatory cell recruitment in vivo. (Blood. 2005;106:3854-3859)

© 2005 by The American Society of Hematology

Introduction

Leukocyte recruitment to inflamed sites is a multistep process consisting of cell tethering, rolling, firm adhesion, transmigration, and retention. Upon inflammatory stimulation, leukocytes in the blood vessel traverse the endothelial cell monolayer and the basement membrane of the blood vessel endothelium and migrate into the interstitial extracellular matrix (ECM). It has been shown that different sets of adhesion receptors are involved at different steps of inflammatory cell recruitment. Members of the integrin family play important roles in several stages of leukocyte migration during inflammation. Integrins are transmembrane receptors composed of α and β heterodimers. To date, 24 distinct integrins assembled from 8 β and 18 α subunits have been described. Both β1 and β2 integrins mediate leukocyte adhesion and migration by interacting with endothelial cells and ECM proteins. Several ECM proteins including fibronectin, vitronectin, collagen, and laminin have been shown to function as ligands for integrins. ECM proteins play important roles in the recruitment of inflammatory cells. Monocytes and neutrophils not only adhere to laminin, thrombospondin, and fibronectin in vitro but also depend on fibronectin, a major ECM component in synovium, for their migration to inflamed sites in rat and mouse arthritis models.

Mandin is a member of the mindin–F-spondin family of ECM proteins. The identified members of this family include murine F-spondin and mindin, zebrafish mindin1 and mindin2, and Droso phila melanogaster M-spondin. All members of the mindin–F-spondin family share 3 domains: FS1 (for F-spondin), FS2, and thrombospondin type 1 repeats. Mouse mindin is expressed abundantly in lymphoid organs and lungs. Mindin functions as a pattern-recognition molecule for microbial pathogens. Mindin-deficient mice exhibit an impaired ability to clear bacterial infection, and mindin-deficient macrophages show defective responses to a broad spectrum of microbial stimuli. Moreover, mindin directly binds to bacteria and their components and functions as an opsonin for the phagocytosis of bacteria.

In this report we have determined the role of mindin in inflammatory cell recruitment in vivo using mindin mutant mice. We found that the recruitment of macrophages and neutrophils was severely impaired in mindin-deficient mice in 4 different inflammation models. We show that neutrophils directly adhere to immobilized mindin matrix. Furthermore, mindin matrix mediates neutrophil migration in response to fMLP (formyl-Met-Leu-Phe), and mindin-mediated migration can be blocked by anti-integrin αM, anti-αδ, and anti-β2 integrin monoclonal antibodies (mAbs). Importantly, HEK-293 cells expressing these integrins exhibit enhanced specific adhesion to coated mindin matrix. Our results suggest that mindin functions as a novel ligand for integrins and plays a critical role in inflammatory cell recruitment.

Materials and methods

Mice

Mindin−/− and mindin+/+ mice were derived from breeding of heterozygous mice after these mice were backcrossed to C57BL/6 for 7 generations and maintained in a specific pathogen-free facility at Duke Vivarium. Six- to 10-week-old age- and sex-matched mice were used in experiments. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Reagents and cell lines

The following blocking antibodies were purchased as listed: CD3 (2C11; Pharmingen, San Diego, CA), CD29 (clone HMβ1-1; Biolegend, San Francisco, CA), and CD45R (clone 30-F11; BD Biosciences, San Jose, CA). Anti-mouse integrin αM (1H8; BioLegend, San Diego, CA), anti-mouse integrin αδ (M290; BioLegend), and anti-mouse integrin β2 (29F1A; BD Biosciences) were used at 25 μg/ml.

Table 1. Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C11</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>30-F11</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>1H8</td>
<td>BioLegend</td>
</tr>
<tr>
<td>M290</td>
<td>BioLegend</td>
</tr>
<tr>
<td>29F1A</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>

From the Department of Immunology, Duke University Medical Center, Durham, NC.


Supported by National Institutes of Health (NIH) grant AI054658 (Y.-W.H.).

W.J. performed most of the experiments in Figures 1 to 5 and analyzed the data; H.L. performed experiments in Figure 1; and Y.-W.H. designed the experiments, analyzed the data, and wrote the manuscript.

Reprints: You-Wen He, Box 3010, Department of Immunology, DUMC, Durham, NC 27710; e-mail: he000004@mc.duke.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology
and then total numbers of leukocytes and neutrophils were counted. For blood collected from anesthetized mice was lysed of red blood cells (RBCs) to determine the number of circulating neutrophils, 50 μL whole blood or bone marrow (BM) cells were incubated with 50 μL 2.4G2 supernatant for 15 minutes on ice followed by anti-Gr-1-Cy5/PE and -CD11b-FITC or other antibodies as indicated. Granulocytes were gated based on side and forward scatter profiles and analyzed for their Gr-1/CD11b expression by flow cytometry.

Neutrophils were purified by discontinuous Percoll (Amersham Bioscience, Arlington Heights, IL) gradients centrifugation as described.20,21 BM or peritoneal cells were layered onto Percoll gradients of 82%/65%/55% and centrifuged at 4°C for 30 minutes. Neutrophils were recovered between the interface of 82%/65%, washed with cold PBS, and resuspended in serum-free Dulbecco modified Eagle medium (DMEM) medium. The purity of neutrophils was approximately 95% as determined by cytospin and H&E staining. Viability determined by 0.1% trypan blue exclusion was greater than 95%.

Neutrophil binding assay
Neutrophil binding assay was performed according to Lowell et al.22 Mindin, fibronectin (Invitrogen), laminin (Sigma), or BSA (Sigma; 100 μL/well at 20 μg/mL) was coated on 96-well plates at 4°C overnight in sodium bicarbonate buffer (pH 9.6). Purified neutrophils from BM or peritoneal cells of wild-type mice were added into the wells and incubated at 37°C for 1 hour. BSA was gently added to each well and then the plates were reversed and left for 5 minutes, then reversed again. Half of the supernatants were discarded right away to remove nonadherent cells. The plates were washed 3 times and centrifuged for 5 minutes. At least 3 different fields of adherent cells in each well were counted under light microscopy.

Neutrophil migration assay
The polycarbonate membranes (pore size 3.0 μm) of transwell for 24-well plates (Corning, Corning, NY) were precoated with 100 μL rMandin, fibronectin, or BSA at 50 μg/mL in PBS overnight at 4°C. DMEM medium (0.6 mL) with or without 100 nM fMLP (Sigma) was added to 96-well plates at 4°C overnight in sodium bicarbonate buffer (pH 9.6). Purified neutrophils from BM or peritoneal cells of wild-type mice were added into the wells and incubated at 37°C for 1 hour. Then the upper chambers were removed and the migrated cells in the lower cell culture plate were counted. For antibody blocking assay, neutrophils purified from mindin−/− BM were mixed with mAbs as indicated and added to the precoated transwell upper chambers. After 1-hour incubation at 37°C, the migrated neutrophils were counted.

Results

Impaired recruitment of inflammatory cells in mindin−/− mice
Our previous results show that mindin−/− mice exhibit defective clearance of GBS infection in the lung.19 The defective clearance of GBS in the mutant mice may result from an impaired activation of macrophages since mindin is critical for macrophage activation.19 However, as an ECM protein, mindin may also play a role in leukocyte recruitment into the lung after GBS infection. To address this issue we determined the number of total cells in BAL fluids from the virus-infected mindin−/− mice 24 hours after GBS infection. To address this issue we determined the number of total cells in BAL fluids from mindin−/− mice 24 hours after GBS infection. The total number of leukocytes in BAL fluids of mindin−/− mice was reduced by more than 70% when compared with wild-type controls (Figure 1A). This reduction was attributed to markedly lower numbers of both macrophages and neutrophils in BAL fluids from mindin−/− mice (Figure 1A). These results demonstrate an important role for mindin in leukocyte migration in the lung after bacterial infection.

We next determined whether mindin plays a similar role in lung infection by viruses. Wild-type and mindin−/− mice were infected with influenza virus strain PR8 (H1N1) intratracheally, and BAL fluids were collected 48 hours after the infection. The total number of leukocytes in BAL fluids from the virus-infected mindin−/− mice was only 30% to 40% of that in control mice (Figure 1B). Furthermore, the recruitment of both macrophages and neutrophils
was significantly impaired (Figure 1B), indicating that mindin is critical for leukocyte recruitment in viral infection.

The above results raise the question whether mindin plays a role in leukocyte recruitment in inflammation caused not only by microbes but also by nonmicrobial stimuli. To test this, we examined leukocyte recruitment in thioglycolate-induced peritonitis in wild-type and mindin<sup>−/−</sup> mice. Peritoneal cells were analyzed at days 1 and 3 for neutrophil and macrophage count, respectively. (A-C) Shown are mean±SD. *P<.05, **P<.001. (D) Number of resident macrophages in the peritoneal cavity of wild-type and mindin<sup>−/−</sup> mice. Resident macrophages were harvested from untreated mice and counted. Numbers are the averages of peritoneal macrophages from each group.

To further examine the role of mindin in inflammatory cell recruitment, we determined neutrophil responses in oxazolone-induced acute dermatitis. Oxazolone was topically applied to the ears of wild-type and mindin<sup>−/−</sup> mice. Neutrophils were harvested from untreated mindin<sup>−/−</sup> mice and counted. Numbers were comparable to that in control mice (Figure 1D). These results suggest that mindin plays a critical role in the recruitment of both neutrophils and macrophages in peritoneal inflammation induced by nonmicrobial stimuli.

We examined whether mindin is also expressed in ear dermis using immunofluorescence staining. Mindin was readily detected in the ear dermis (Figure 2A). As a negative control, ear dermis from mindin<sup>−/−</sup> mice displayed no detectable staining (Figure 2B). Taken together, our results demonstrate that mindin is critical for the recruitment of neutrophils and macrophages to inflammation sites in 4 different inflammation models.

The lack of recruited macrophages in inflammation sites in mindin<sup>−/−</sup> mice is not due to defective macrophage development (Figure 1D and He et al<sup>19</sup>). We also examined neutrophil development in mindin<sup>−/−</sup> mice. The number of neutrophils in the peripheral blood of mindin<sup>−/−</sup> mice was comparable to that of control mice (Figure 3A). Phenotypic analysis revealed that...
and control neutrophils expressed similar levels of Gr-1, a neutrophil surface marker (Figure 3B). Furthermore, the expression levels of various integrins involved in leukocyte migration were not obviously changed on mindin null neutrophils (Figure 3C).

 Taken together, these results demonstrate that mindin is not required for neutrophil development and the normal expression of various integrins.

**Mindin mediates neutrophil migration in vitro**

Mindin, as an ECM protein, may directly interact with receptors expressed on neutrophils and macrophages and provide adhesion sites for leukocyte migration. To determine whether neutrophils can directly adhere to immobilized mindin, we incubated purified neutrophils from wild-type mice with coated mindin. Two other ECM proteins, laminin and fibronectin, were used as positive controls. BSA was used as a control for baseline adhesion. BM neutrophils displayed slightly increased binding to immobilized mindin and laminin over BSA but strong adhesion to fibronectin (Figure 4A). The weak neutrophil adhesion to mindin may be related to their nonactivated status. We then activated BM neutrophils with PMA and tested their binding to mindin matrix. As shown in Figure 4B, activated BM neutrophils displayed enhanced adhesion to mindin and laminin matrix. We also examined the adhesion of activated neutrophils from inflamed peritoneum. Neutrophils from thioglycollate-induced peritoneal cells displayed a 3- to 4-fold enhanced binding to coated mindin as well as laminin, similar to that exhibited by fibronectin (Figure 4C).

These results demonstrate that activated neutrophils adhere to immobilized mindin.

We next assessed whether mindin can promote neutrophil migration in a transwell-migration assay. Mindin and fibronectin were coated on the membranes of transwells. Neutrophils were induced to migrate through the membranes with fMLP. Both mindin and fibronectin enhanced fMLP-induced neutrophil migration by 2- to 3-fold, whereas BSA had no such effect (Figure 4D). Moreover, neutrophils purified from mindin null mice behaved similarly to those from wild-type mice in the binding and migration assays (Figure 5A; data not shown). These results suggest that mindin promotes neutrophil migration through adhesion rather than regulating neutrophil cellular function.
Neutrophils interact with mindin through integrins

Like many other ECM proteins, mindin may mediate adhesion of neutrophils through integrins. To test this, we examined the effect of several anti-integrin mAbs on IMLP-induced neutrophil migration through mindin-coated membranes. Among the 6 antibodies tested, anti-β1 (CD29), -α4 (CD49e), and -αε (CD11a) integrin mAbs did not have a significant effect on mindin-mediated neutrophil migration (Figure 5A). In contrast, anti-αζ (CD49d), -αδ (CD11b), and -β2 (CD18) integrin mAbs inhibited neutrophil migration in this assay (Figure 5A). Anti-αζ integrin mAb blocked mindin-mediated neutrophil migration to the baseline level mediated by BSA, whereas anti-αδ and -β2 mAb treatment decreased mindin-specific neutrophil migration by 50% to 60% (Figure 5A). These results suggest that mindin may serve as a ligand for integrins αε, αζ, and β2 in neutrophil recruitment.

To further assess integrin-mindin interaction, we transiently cotransfected HEK-293 cells with expression plasmids encoding αδ, β2, or αε and β1 integrins to achieve cell surface integrin expression. FACS analysis revealed that 60% to 70% of transfected HEK-293 cells expressed the appropriate integrins (Figure 5B). We first determined the adhesion of unstimulated HEK-293 cells transfected with αδβ2 or αεβ1 integrins to immobilized mindin and BSA. We did not observe significant increase in cell binding to mindin over BSA (data not shown). We then activated the HEK-293 cells with PMA and tested for their adhesion. Expression of αδβ2 or αεβ1 integrins enhanced HEK-293 cell binding to mindin by 3- to 4-fold (Figure 5C). In contrast, vector-transfected and parental HEK-293 cells did not display any increased binding to mindin after PMA activation. Interestingly, the enhanced adhesion of HEK-293 cells expressing αδβ2 or αεβ1 integrins to mindin was blocked by all 4 anti-integrin mAbs, including anti-β1 (Figure 5C). In contrast, anti-CD3 mAb serving as a negative control did not affect the binding of HEK-293 cells expressing αδβ2 or αεβ1 integrins to mindin (Figure 5C). As expected, the anti-integrin mAbs blocked the binding of the transfected HEK-293 cells to fibronectin (Figure 5C). Taken together, these results demonstrate that mindin specifically interacts with αε, αδ, β1, and β2 integrins.

Discussion

In this report, we demonstrate that the ECM protein mindin has a critical role in inflammatory cell recruitment in vivo and functions as a ligand for integrins in vitro. Leukocyte recruitment to inflamed sites depends on the interaction of various integrins expressed on leukocytes and their ligands expressed on endothelial cells or in the interstitium. The roles of integrins in macrophage and neutrophil extravasation during inflammation have been extensively investigated.5,7,22-26 Numerous studies have also demonstrated that ECM proteins, such as laminin, thrombospondin, and fibronectin, contribute to neutrophil and macrophage adhesion and migration in vitro and in vivo by functioning as integrin ligands.9,14,27,28 Our data have added another ECM molecule into the list of an increasing number of proteins with an important function in inflammatory cell recruitment.

The defective inflammatory cell recruitment in mindin-deficient mice likely reflects a combined effect of an impaired in vivo interaction between mindin in the interstitium and integrin αε, β1, and αδ expressed on neutrophils and macrophages as well as an impaired activation of innate immune cells. Although our previous data demonstrate that mindin functions as a pattern-recognition molecule for microbial pathogens and that mindin-deficient macrophages and mast cells have defective activation by microbial pathogens,19 the impaired inflammatory cell recruitment in mindin-deficient mice is not solely due to a secondary effect of impaired innate cell activation. Several lines of evidence support the notion that mindin has a direct role in leukocyte migration in vivo by functioning as a ligand for integrins during inflammatory cell recruitment. First, neutrophils directly adhere to mindin matrix, and mindin can mediate neutrophil migration in transwell migration assays. Second, mindin-mediated neutrophil migration can be blocked by anti-αε, -αδ, and -β2 mAbs. Third, HEK-293 cells expressing these integrins exhibit specific adhesion to mindin matrix. Fourth, importantly, mindin-deficient mice exhibit defective neutrophil and macrophage cell recruitment during inflammations induced by nonpathogenic stimuli.

The roles of integrin αε and αδβ2 in inflammatory cell recruitment have been firmly established.22,23,25,26,29-33 The αε and αδβ2 integrins play different roles in leukocyte recruitment, with αδβ2 mediating adhesion of leukocytes to endothelium and αε interacting with ECM. Nevertheless, it appears that mice deficient for β2 integrin exhibit only a partial defect in neutrophil recruitment,22,23,25,26,33,34 suggesting a β1 integrin–dependent pathway for neutrophil recruitment in which αε integrin may play a major role. Therefore, impaired inflammatory cell recruitment in mindin-deficient mice may result from a lack of interactions between these several integrins and mindin in the ECM. In addition, mindin may also serve as a ligand for other integrins that also play important roles in inflammatory cell recruitment. It is interesting to note that while the binding of neutrophils to mindin was not blocked by anti-β1 integrin mAb, the binding of β1 integrin–transfected HEK-293 cells to mindin was blocked by the same mAb. This differential effect by anti-β1 mAb suggests that β1 integrin may assume different configurations on neutrophils and HEK-293 cells.

Why do other ECM ligands for αεβ1 and αδβ2 integrins, such as fibronectin, fail to compensate for mindin deficiency in vivo? This may be related to the binding properties of these integrins. Integrins including αε and αδβ2 display low-affinity and high-affinity binding depending on their state of activation.34 These integrins exhibit low-affinity binding in their default conformation in resting leukocytes. When activated by so-called inside-out signaling, integrins display high-affinity binding via conformational changes rather than an increase of their expression levels. We observed that the binding to mindin by neutrophils and transfected HEK-293 cells was at a minimum level without activation. However, activation of these cells with PMA markedly increased their binding to mindin. In contrast, consistent with previous data, PMA activation of neutrophils did not enhance their binding to fibronectin.28,35 These results suggest that the adhesive binding of neutrophils in vivo may require their interaction with multiple ECM proteins including fibronectin and mindin. Neutrophils only interact with mindin in a high-affinity state after activation during an inflammation. This type of interaction is unique for mindin and cannot be compensated by other ECM ligands. Alternatively, mindin may serve as a ligand for other unidentified adhesion receptors.

Our results raise an interesting question whether αε, αδ, β1, and β2 integrins serve as coreceptors for recognition of microbial pathogens in macrophages and mast cells. Mindin-deficient macrophages have defective responses to a broad spectrum of microbial stimuli.19 Microbial pathogens may be recognized on one hand by Toll-like receptors (TLRs) and on the other hand interact with integrins through mindin. Both types of interactions may be required to efficiently activate innate immune cells. This notion is
supported by recent works showing that integrin α4β1 forms clusters with TLRs and other pattern recognition receptors in lipid rafts of activated cells. Furthermore, in cooperation with CD14 and TLR2, integrin α4β1 participates in the recognition of bacterial fimbriae. Future studies on the roles of integrins in pathogen recognition will shed novel insights on innate immune mechanisms.

Acknowledgments
We thank Dr Michael Dee Gunn for help in migration assay and Drs Gulgilemo Venturi, Jonathan Poe, and Heather Hartig for critical review of this manuscript.

References
The extracellular matrix protein mindin serves as an integrin ligand and is critical for inflammatory cell recruitment

Wei Jia, Hong Li and You-Wen He