Conditional deletion of FAK in mice endothelium disrupts lung vascular barrier function due to destabilization of RhoA and Rac1 activities

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Schmidt TT, Tauseef M, Yue L, Bonini MG, Gothert J, Shen T, Guan J, Predescu S, Sadikot R, Mehta D. Conditional deletion of FAK in mice endothelium disrupts lung vascular barrier function due to destabilization of RhoA and Rac1 activities. Am J Physiol Lung Cell Mol Physiol 305: L291–L300, 2013. First published June 14, 2013; doi:10.1152/ajplung.00094.2013.—Loss of lung-fluid homeostasis is the hallmark of acute lung injury (ALI). Association of catenins and actin cytoskeleton with vascular endothelial (VE)-cadherin is generally considered the main mechanism for stabilizing adherens junctions (AJs), thereby preventing disruption of lung vascular barrier function. The present study identifies endothelial focal adhesion kinase (FAK), a nonreceptor tyrosine kinase that canonically regulates focal adhesion turnover, as a novel AJ-stabilizing mechanism. In wild-type mice, induction of ALI by intraperitoneal administration of lipopolysaccharide or cecal ligation and puncture markedly decreased FAK expression in lungs. Using a mouse model in which FAK was conditionally deleted only in endothelial cells (ECs), we show that loss of EC-FAK mimicked key features of ALI (diffuse lung hemorrhage, increased transvascular albumin influx, edema, and neutrophil accumulation in the lung). EC-FAK deletion disrupted AJs due to impairment of the fine balance between the activities of RhoA and Rac1 GTPases. Deletion of EC-FAK facilitated Rhoa’s interaction with p115-RhoA guanine exchange factor, leading to activation of Rhoa. Activated Rhoa antagonized Rac1 activity, destabilizing AJs. Inhibition of Rho kinase, a downstream effector of RhoA, reestablished normal endothelial barrier function in FAK−/− ECs and lung vascular integrity in EC-FAK−/− mice. Our findings demonstrate that EC-FAK plays an essential role in maintaining AJs and thereby lung vascular barrier function by establishing the normal balance between RhoA and Rac1 activities.

focal adhesion kinase; adherens junctions; acute lung injury; endothelial barrier

THE VASCULAR ENDOTHELIUM CONTROLS the passage of macromolecules and fluid between the blood and interstitial space and thereby plays a vital role in maintaining tissue-fluid homeostasis (20). It is known that loss of endothelial barrier function results in tissue edema, the hallmark of acute lung injury (ALI), which induces ~40% mortality in affected patients (18, 20, 28). Homotypic interaction between vascular endothelial (VE)-cadherin from contiguous endothelial cells forms adherens junctions (AJs) that primarily maintain endothelial barrier function (20). While VE-cadherin linkage with intracellular catenins (α, β, and p120) and actin cytoskeleton is a well-accepted mechanism for stabilizing AJs, additional cellular molecules may be required.

Focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, regulates endothelial cell-matrix attachment (20, 27). FAK may also maintain AJs by converging on multiple signaling pathways, but this remains controversial (20). We showed that FAK mediates the interaction of p120-catenin with actin-binding machinery by phosphorylating neural-Wiscott Aldrich syndrome protein that stabilizes AJs (15, 25). FAK also suppressed the activity of the small GTPase RhoA to restrict endothelial contraction (13). FAK thereby maintained basal endothelial barrier function and induced resealing of endothelial junctions following the increase in endothelial permeability by thrombin (13, 15, 21). Similarly, Quadri et al. showed that FAK prevents oxidant-induced barrier dysfunction by regulating AJs function (23, 24). Also small-interfering RNA (siRNA)-induced depletion of FAK or expression of dominant-negative FAK impaired AJ formation and enhancement of barrier function by sphingosine-1-phosphate and lysophosphatidic acid (12, 30). However, other studies showed that dominant-negative FAK or a kinase dead FAK mutant prevented AJ disruption in response to VEGF or oxidants, indicating that FAK in fact disrupts barrier function (6, 41, 46). Endothelial-specific deletion of FAK in mice induced embryonic lethality (3, 29). Thus, it remains enigmatic whether FAK preserves lung vascular barrier function and how FAK attains it.

In the present study, we conditionally induced FAK deletion in the endothelium of mice, referred to as EC-FAK−/− mice hereafter, to address this question fundamental to regulation of vascular barrier function. We demonstrate that endothelial FAK deletion spontaneously disrupts lung endothelial barrier function. We show that loss of FAK results in disruption of AJs due to impairment of the fine balance between the activities of RhoA and Rac1 GTPases.

MATERIALS AND METHODS

Materials. Human pulmonary arterial endothelial cells (HPAECs), endothelial growth medium (EBM-2), Nucleofector kit, and Amaxa electroporation kit were obtained from Lonza. Secondary fluorescent
Fig. 1. Focal adhesion kinase (FAK) protein expression is decreased during acute lung injury in mice. Lungs lysates from wild-type mice exposed to vehicle or lipopolysaccharide (LPS, 30 mg/kg, 6 h) (A) or subjected to cecal ligation (control) or cecal ligation and puncture (CLP, 22 h) (B) were immunoblotted with anti-FAK or anti-β-actin (loading control) antibodies (Abs). Bar graphs show the densitometric analysis of FAK expression from 4 lungs/group (A and B). *Statistically significant difference from vehicle or control lungs (P < 0.05).

Fig. 2. Inducible deletion of endothelial FAK. A: schematic of tamoxifen-induced deletion of FAK in Cre-expressing FAK\textsuperscript{fl/fl} mice. Four-week-old FAK\textsuperscript{fl/fl} (control) and FAK\textsuperscript{floxed}, expressing Cre mice were injected with 2 mg tamoxifen ip for 5 consecutive days, followed by a rest period of 5 days. Mice were used for experiments on the 11th day. B: genotype showing tamoxifen deletion of FAK. Tails from FAK\textsuperscript{fl/fl} or endothelial cell (EC)-FAK\textsuperscript{null} mice were digested, and FAK and Cre expression was determined using specific primers. Cre induces FAK deletion in Cre-expressing EC-FAK\textsuperscript{null} lungs from 5 mice/group. *Statistical significance at the 0.05 level.

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**Heme quantification.** Heme from lung supernatants was quantified using a QuantiChrom heme assay kit (Bioxys/Gentaur).

**Assessment of lung capillary leakage.** Evans blue-labeled albumin extravasation in lung parenchyma and lung wet-to-dry weight ratio were used as indexes of lung vascular barrier function (15, 37).

**Myeloperoxidase assay.** Myeloperoxidase activity was measured as described (1). Data are represented as change in absorbance at 450 nm over a 5-min period following addition of H2O2 per gram lung weight.

**RhoGTPase activities and immunoblotting.** Lysates were incubated with either PAK-PBD or Rhotekin-PBD beads to determine RhoGTPase activities (13, 37). Where indicated, cell monolayers were...
incubated with 20 μM SB-203580, 10 μM Y-27632, or DMSO (vehicle) for 30 min before experiment.

Endothelial monolayer permeability. Monolayer permeability was determined by measuring transendothelial influx of Evans blue-labeled albumin (37).

Statistical analysis. Statistical comparisons were made using ANOVA followed by two-tailed Student's t-test. Differences were considered significant at P < 0.05.

RESULTS

Lung injury is associated with decreased FAK expression in marine lungs. To assess whether increased endothelial permeability is associated with decreased FAK expression, we used two well-characterized mouse models of ALI (1, 38). We injected a sublethal dose (30 mg/kg) of LPS intraperitoneally or performed CLP. Intriguingly, both LPS and CLP induced a ~40% decrease in FAK protein expression (Fig. 1, A and B), indicating that FAK expression is decreased during lung injury.

Endothelial cell-specific FAK deletion impairs lung fluid balance. To determine whether the decreased endothelial expression of FAK seen in the above studies is causally related to impairment of lung vascular barrier function, we generated mice in which FAK deletion was conditionally induced in endothelial cells. We crossed FAK-floxed mice (FAKfl/fl) (29) with SCL-Cre-ER TG mice. Scl-Cre-ER TG mice express Cre recombinase in endothelial cells driven by the SCL enhancer region (1). FAK deletion was induced by tamoxifen administration at 4 wk of age (Fig. 2A). We also subjected aged-matched FAKfl/fl mice to identical tamoxifen treatment to control for its nonspecific effects on endothelium. Recombination of the FAK gene was detected in Cre-expressing FAKfl/fl mice (EC-FAK ko/kg), whereas no recombination was detected in mice lacking the Cre transgene (FAKfl/fl) (Fig. 2B). Tamoxifen-induced Cre recombinase activity markedly decreased FAK mRNA and protein expression in EC-FAK ko/kg lung lysates (Fig. 2C). Next, we communostained lung sections obtained from EC-FAK ko/kg or FAKfl/fl mice with anti-FAK and anti-CD31 (an endothelial cell marker) Abs to determine specific deletion of FAK in the endothelium. As expected, FAKfl/fl lung sections revealed homogenous FAK staining in CD31 + cells (Fig. 2, D and E). However, EC-FAK ko/kg lungs barely showed FAK staining in CD31 + cells (Fig. 2, D and E). In EC-FAK ko/kg lungs, tamoxifen did not alter FAK expression in epithelial or smooth muscle cells (Fig. 2F) or in hematopoietic cells (Fig. 2G).

Detectable FAK expression in EC-FAK ko/kg lung lysates reflected FAK expression in nonendothelial cells, since Cre recombinase activation completely deleted FAK in LECs isolated from FAKfl/fl mice (Fig. 2H).

Intriguingly, we observed diffuse hemorrhage in EC-FAK ko/kg lungs as indicated by the threefold increase in heme levels (Fig. 3A). Additionally, endothelial FAK deletion spontaneously increased lung edema formation as demonstrated by significant increases in transvascular albumin influx and lung wet-to-dry weight ratio in EC-FAK ko/kg lungs (Fig. 3, B and C). We confirmed that tamoxifen injection alone had no effect on lung vascular permeability or lung wet-to-dry weight ratio in wild-type (WT), Cre, or FAKfl/fl mice (Fig. 3, D and E).

Hematoxylin and eosin staining of EC-FAK ko/kg lungs revealed a threefold increase in lung leukocyte infiltration and a twofold increase in tissue myeloperoxidase activity (Fig. 3, F and G). Also, deletion of FAK did not alter the mRNA expression of related nonreceptor tyrosine kinases, including Fyn, Src, and Pyk2 (Fig. 3H).

To corroborate the above studies in lungs, we assessed the integrity of FAK−/− endothelial monolayers by determining VE-cadherin and actin organization. FAK deletion impaired cell-surface VE-cadherin localization (Fig. 3I, top) and increased actin stress fiber formation (Fig. 3I, bottom) and MLC phosphorylation (Fig. 3J), resulting in a sixfold elevation in interendothelial gap area (Fig. 3K). Total expression of VE-cadherin, p120-catenin, and MLC proteins was not altered in FAK ko/kg ECs (Fig. 3J).

FAK deletion disrupts balance between RhoA and Rac1 GTPase activities. It is well known that RhoA and Rac1 play a key role in maintaining AJ strength (2, 4, 43–45). Thus, we asked whether deletion of FAK compromises endothelial barrier function by modulating RhoA and Rac1 activities. We observed that FAK null endothelial cells showed a twofold increase in RhoA activity (Fig. 4A), but, crucially, the activity of Rac1 was concomitantly decreased by a factor of approximately five (Fig. 4A). Similarly, reduction of FAK levels in HPAECs by 90% using siRNA inactivated Rac1 while inducing RhoA activity (Fig. 4B).

Next, we inhibited RhoA signaling using the Rho kinase (ROCK) inhibitor Y-27632 (9, 44) to assess whether activated RhoA was responsible for suppression of Rac1 activity in FAK-depleted ECs. Whereas inhibition of ROCK, which we confirmed by determining the phosphorylation of myosin-binding subunit of myosin phosphatase, modestly increased Rac1 activity in ECs transfected with scrambled siRNA (Fig. 4C), it resulted in an eightfold increase in Rac1 activity in FAK-depleted ECs (Fig. 4C).

RhoA activity is regulated by the GDP-GTP exchange cycle induced by guanine exchange factors (GEFs) such as p115RhoGAP and p190Rho guanine-activating protein (p190RhoGAP) (14, 20, 35). Because p190RhoGAP was shown to require FAK for full guanine-activating protein (GAP) activity (13), we tested the hypothesis if loss of FAK facilitated the complex formation between p115RhoGAP and RhoA, required for RhoA activation (14). Cell lysates from FAK-knockdown cells were immunoprecipitated with anti-RhoA Ab followed by immunoblotting with anti-p115RhoGAP Ab to assess their interaction. We found that RhoA barely interacted with p115RhoGAP in control cells, but, importantly, the interaction of p115RhoGAP with RhoA markedly increased in FAK knockdown ECs (Fig. 4D). No interaction of RhoA was observed with control IgG (Fig. 4D).

Inhibition of RhoA reinstates lung-fluid balance in EC-FAK ko/kg mice. We transduced WT-FAK cDNA in FAK null lungs using liposomes (15, 38) to assess whether restoration of FAK expression in EC-FAK null mice suppressed edema formation. WT mice injected with liposomes containing vector were used as controls. Restoration of FAK in EC-FAK ko/kg mice significantly reduced edema formation (Fig. 5A).

To address whether increased RhoA activity was required and sufficient for increasing lung vascular leak in EC-FAK null mice, we inhibited RhoA signaling in FAK null ECs and EC-FAK ko/kg mice and assessed changes in endothelial permeability. RhoA inhibition in FAK ko/kg ECs restored basal endothelial permeability (Fig. 5B) and reinstated lung-fluid balance in EC-FAK ko/kg mice (Fig. 5C).
DISCUSSION

We have demonstrated in these studies that endothelial FAK is required for stabilizing AJs and thereby maintains lung-vascular barrier function. We showed that induction of ALI in mice decreases FAK protein expression in the lungs. Importantly, conditional deletion of FAK in endothelial cells facilitated the interaction of p115RhoGEF with RhoA enabling RhoA antagonism of Rac1 activity which in turn disrupted AJs...
leading to an increase in endothelial permeability. Therefore, our findings identify for the first time a novel role of endothelial FAK in maintaining AJs in pulmonary endothelium by determining the normal balance between RhoA and Rac1 activities.

ALI is a leading cause of death after sepsis (18, 28). Increased endothelial permeability is known to be the primary cause of ALI (18, 20, 28). FAK plays a key role in regulating endothelial permeability in response to several edemagenic mediators (20, 39). However, whether sepsis induces ALI by altering FAK expression remains unclear. We showed that FAK protein expression is decreased following sepsis in murine lungs. Intriguingly, conditional deletion of FAK in mouse endothelial cells induced lung vascular barrier disruption and leukocyte infiltration in the interstitium, recapitulating key features of ALI. Decreased endothelial FAK expression is therefore a likely factor responsible for the pulmonary vascular hyperpermeability and edema formation seen during ALI.
mechanism by which FAK is degraded during ALI in the endothelium remains to be parsed out. LPS activates caspases and calpain (36). FAK contains caspase- and calpain-binding sites (5, 16), making FAK susceptible to degradation during ALI, thereby leading to vascular dysfunction.

AJs formed by intercellular interaction of VE-cadherin primarily regulate endothelial permeability (20). Association of catenins and actin cytoskeleton with VE-cadherin is generally considered the main mechanism for maintaining AJ stability (20), thereby preventing disruption of lung vascular barrier function. FAK has been shown to regulate several endothelial cell functions, such as migration, proliferation, and angiogenesis, by regulating the turnover of focal adhesions (20). Our findings showing that FAK deletion markedly impairs cell-surface VE-cadherin expression and elevates actin stress fiber as well as interendothelial gap formation subsequently leading to lung vascular leak in EC-FAK null mice provide unequivocal evidence that FAK is required to maintain AJs. Consistently, studies in endothelial cells have shown that impairment of FAK function also disrupted AJs (13, 15, 21, 23–25, 30, 34). Thus, we conclude that FAK prevented the loss of lung vascular barrier by maintaining AJs.

The monomeric RhoGTPases RhoA and Rac1 play a central role in regulating the integrity of AJs (20). Permeability-increasing mediators rapidly activate RhoA, which mediates stress fiber formation and disruption of AJs (4, 13, 15, 44). In

Fig. 4. FAK deletion impairs normal balance between RhoA and Rac1 activities. A and B: effect of FAK deletion on RhoA and Rac1 activities. FAK−/− and FAK+/+ LECs were lysed, and the activity of RhoA or Rac1 was determined using rhotekin or PAK pull down beads (A). Data represent means ± SD of GTPase activities compared with those in FAK+/+ LECs. *Significant difference from FAK+/+ LECs (P < 0.05; n = 3). Human pulmonary artery endothelial cells (HPAECs) transfected with scrambled (siSc) or FAK (siFAK) small-interfering RNA (siRNA) were lysed to determine RhoA and Rac1 activities (B). In parallel, lysates were immunoblotted with anti-FAK and anti-actin Abs to assess FAK depletion in ECs using actin as a loading control (inset). Blot is representative of findings from three individual experiments. *Significant difference from siSc-transfected HPAECs (P < 0.05; n = 3). C: RhoA inhibits Rac1 activity. Scrambled and FAK siRNA-expressing HPAECs were incubated with 10 μM Y-27632 to inhibit Rho kinase (ROCK), a downstream effector of RhoA. After 30 min, cells were lysed, and Rac1 activity was determined to assess whether inhibition of ROCK restores normal Rac1 activity. *Significant difference from siSc-transfected HPAECs (P < 0.05; n = 3). In bar graph, Rac1 activity in Y-27632-pretreated siSc- or siFAK-transfected cells is plotted relative to their respective vehicle-treated values to better discern the level of restoration seen in siFAK cells after inhibiting ROCK. *Significant difference from siSc-transfected HPAECs or vehicle-treated siFAK cells (P < 0.05; n = 3). D: FAK depletion promotes RhoA interaction with p115RhoGEF. Lysates from scrambled and FAK siRNA-expressing HPAECs were immunoprecipitated with either control IgG or anti-RhoA antibody followed by immunoblotting with anti-p115RhoGEF and anti-RhoA Abs. The blot shown is representative of data from multiple experiments. *Significant difference from siSc-transfected HPAECs (P < 0.05; n = 4).
Fig. 5. Blockade of RhoA reinstates lung vascular permeability. A: restoration of FAK expression in EC-FAK−/− mice rescues lung vascular permeability. FAK−/− or EC-FAK−/− mice expressing indicated constructs were assessed for lung edema formation by determining lung wet-to-dry weight ratios. Graph represents means ± SD. *Significance from GFP or GFP-FAK transducing FAK−/− lungs or GFP-FAK transducing EC-FAK−/− lungs (P < 0.05, n = 4). Inset shows representative immunoblot of free GFP or GFP-FAK fusion protein found in lung lysates from EC-FAK−/− mice with the aid of an anti-GFP antibody. A representative immunoblot from 4 mouse lungs/group is shown. All experiments were performed 48 h after injection of liposome-encapsulated mutants. B and C: inhibition of RhoA signaling rescues endothelial barrier function in FAK-depleted ECs and lungs. B: FAK+/+ or FAK−/− LEC monolayers were pretreated with vehicle or 10 µM ROCK inhibitor Y-27632 to inhibit ROCK. After 30 min, transendothelial albumin influx was determined using Evans blue-conjugated albumin. Experiments were performed in duplicate at least three times. *Significant difference from FAK−/− LECs (P < 0.05). C: FAK−/− or EC-FAK−/− mice were retro-orbitally injected with either vehicle or ROCK inhibitor Y-27632 (10 mg/kg). Mice were killed after 30 min, and lung wet-to-dry weight ratio was calculated. *Significance from PBS- or Y-27632-exposed FAK−/− lungs or EC-FAK−/− lungs receiving Y-27632 (P < 0.05; n = 3–4 mice/group). D: model of endothelial FAK regulation of lung vascular permeability described in text.
In summary, we showed that FAK expression is significantly decreased in the setting of two well-established murine models of ALI. Conditional loss of FAK in the mouse endothelium leads to hyperactivation of RhoA, which antagonizes Rac1 activity, leading to disruption of AJs and loss of lung vascular barrier function, a characteristic feature of ALI. In this context, normalizing the level of FAK expression in endothelial cells after sepsis may be an attractive pharmacological approach to preventing sepsis-induced lung injury.

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DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS


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