ADAM15 regulates endothelial permeability and neutrophil migration via Src/ERK1/2 signalling

Chongxiu Sun¹, Mack H. Wu¹, Mingzhang Guo¹, Mark L. Day², Eugene S. Lee¹, and Sarah Y. Yuan¹*

¹Division of Research, Department of Surgery, University of California Davis School of Medicine, 4625 2nd Avenue, Room 3006, Sacramento, CA 95817, USA; and ²Department of Urology, University of Michigan, Ann Arbor, MI 48109, USA

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Aims

Endothelial barrier dysfunction is a key event in the pathogenesis of vascular diseases associated with inflammation. ADAM (a disintegrin and metalloprotease) 15 has been shown to contribute to the development of vascular inflammation. However, its role in regulating endothelial barrier function is unknown. The aim of this study was to examine the effect of ADAM15 on endothelial permeability and its underlying mechanisms.

Methods and results

By measuring albumin transendothelial flux and transendothelial electric resistance in cultured human umbilical vein endothelial cell monolayers, we found that depletion of ADAM15 expression via siRNA decreased endothelial permeability and attenuated thrombin-induced barrier dysfunction. In contrast, endothelial cells overexpressing either wild-type or catalytically dead mutant ADAM15 displayed a higher basal permeability and augmented hyperpermeability in response to thrombin. In addition, ADAM15 knockdown inhibited whereas ADAM15 overexpression promoted neutrophil transendothelial migration. Further molecular assays revealed that ADAM15 did not cleave vascular endothelial-cadherin or cause its degradation. However, overexpression of ADAM15 promoted extracellular signal-regulated kinase (ERK)1/2 phosphorylation in both non-stimulated and thrombin-stimulated endothelial cells in a protease activity-independent manner. Pharmacological inhibition of Src kinase or ERK activation reversed ADAM15-induced hyperpermeability and neutrophil transmigration.

Conclusion

The data provide evidence for a novel function of ADAM15 in regulating endothelial barrier properties. The mechanisms of ADAM15-induced hyperpermeability involve Src/ERK1/2 signalling independent of junction molecule shedding.

Keywords

Endothelial permeability • Metalloproteinase • Vascular inflammation • Signal transduction

1. Introduction

Vascular endothelial (VE) cells constitute a semi-permeable barrier that controls the transport of fluid, solutes, and cells across the vessel wall. Endothelial hyperpermeability is considered one of the most important cellular processes in the development of inflammatory disorders, such as sepsis,¹ atherosclerosis,² and diabetic complications.³ The mechanisms underlying permeability regulation have been the subject of extensive investigation. It is commonly recognized that cell–cell adhesion and integrin-mediated cell–matrix interactions play a critical role in the maintenance of barrier function as well as in the mediation of responses to inflammatory stimuli such as thrombin.⁴

As an important type of cell–cell junctions in the peripheral vascular endothelium, the adherens junction is mainly composed of VE-cadherin and catenins. VE-cadherin mediates homophilic binding of adjacent cells via its extracellular domain and associates with catenins via its cytoplasmic tail, thereby linking the junction complex to the cytoskeleton.⁵ Disruption of the junction leads to increased vascular permeability; an effect seen in diseased vasculature with elaborated inflammatory mediators, including thrombin,⁶ cytokines, and VE growth factor.⁷ Moreover, the adherens junction participates in the regulation of leucocyte extravasation. For example, VE-cadherin disruption has been shown to facilitate monocyte transendothelial migration.⁸ Likewise, blocking VE-cadherin with antibodies promotes neutrophil migration.⁹ In addition to cadherin-mediated cell–cell

* Corresponding author. Tel: +1 916 703 0422; fax: +1 916 703 0421, Email: sarahyuan@ucdavis.edu
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ADAM15 signaling in endothelial permeability

interactions, integrin binding to the extracellular matrix strengthens endothelial barrier function. Competitive disruption of integrin-matrix interactions with RGD peptides causes endothelial hyperpermeability. ADAMs (a disintegrin and metalloprotease) are a family of type I transmembrane glycoproteins that have been implicated in inflammation, angiogenesis, and cancer growth and metastasis. Structurally, each ADAM molecule consists of a prodomain, a metalloproteinase domain, a disintegrin domain, a cystein-rich domain, and a cytoplasmic tail. The metalloproteinase domain has a consensus HEXXGXXH sequence capable of degrading matrix and shedding cytokines, growth factor and growth factor receptors, and cadherins. The disintegrin domain of human ADAM15 contains an RGD sequence that can regulate integrin binding and subsequent cell–cell and cell–matrix adhesions. In addition to the proteolytic and adhesive properties, ADAM15 possesses a cytoplasmic tail with potential recognition sites for several protein kinases that are known to be major players in signal transduction. These unique features provide a structural basis for ADAM15 to exert pleiotropic functions via distinct mechanisms. In this study, we tested whether protease shedding of VE-cadherin, disintegrin-mediated disruption of focal adhesion, or intracellular signal transduction via Src and extracellular signal-regulated kinase (ERK) 1/2 played a relatively important role in ADAM15-induced permeability responses in human endothelial cells in the presence and absence of inflammatory stimulation by thrombin.

We also examined the effect of altered ADAM15 expression on endothelial permeability because recent studies indicate that ADAM15 upregulation is functionally involved in pathological angiogenesis and tumour metastasis. Moreover, ADAM15 is upregulated in cells treated with pro-inflammatory cytokines and in tissues of inflammatory diseases such as atherosclerosis, rheumatoid arthritis, and irritable bowels, indicating the possibility that increased ADAM15 serves as an inflammatory mediator. However, the specific role of ADAM15 in regulating VE permeability has not been established, and even less is known about its mechanism of action. Therefore, characterization of the molecular basis underlying ADAM15-induced permeability responses would be important for a further understanding of inflammatory diseases.

2. Methods

2.1 Transfection
Plasmid pcDNA/human WT ADAM15 with haemagglutinin tag at the C-terminus was generated as previously described. Mutant ADAM15 cDNA producing a catalytically dead metalloproteinase was constructed by mutating the glutamate into alanine at 350 (E350A). Human umbilical vein endothelial cells (HUVECs) (Combrex, MD, USA) were maintained to confluence. Then phospho (Thr202/Tyr204)- and total ERK1/2 were monitored with specific antibodies (Cell Signaling, MA, USA). To test VE-cadherin shedding, cells were treated with the γ-secretase inhibitor DAPT (10 μM) for 16 h followed by western blotting using an anti-VE-cadherin C-terminus antibody (Santa Cruz, CA, USA). Cells were lysed with RIPA buffer and protein concentration determined by BCA assay. Cell lysates corresponding to 20 μg protein were submitted to western blotting as previously described. When necessary, stripping buffer (Thermo, VT, USA) was applied to the same membrane for reprobing.

2.3 Albumin transendothelial flux
Albumin flux across cultured HUVEC monolayers was measured as previously described. Briefly, 10^5 HUVECs were grown to confluence on a transwell membrane (Corning, NY, USA). FITC-labelled albumin (15 μM) was added to the top chamber in the presence or absence of 25 nM thrombin. After 1 h incubation, concentrations of albumin in the top and bottom chambers were monitored with a fluorescence microplate reader (Bio-Tek, VT, USA). The permeability coefficient of albumin (Pa) was determined as Pa = [A]/t (1/A x V/L), where [A] is the bottom chamber concentration, t the time (s), A the area of the membrane (cm²), V the bottom chamber volume, and [L] the top chamber concentration. At the same time, parallel wells were submitted to MTT (Roche, CA, USA) assay and Calcein AM (Invitrogen, CA, USA) staining to confirm cell quantity and confluence of the monolayers.

2.4 Transendothelial electrical resistance (TER)
As an indicator of cell–cell and cell–matrix adhesive properties, transendothelial electrical resistance (TER) was measured as previously described. Briefly, 10^5 cells were seeded onto ECIS electrode arrays (Applied Biophysics, NY, USA) and grown overnight. With a 1 V, 4000 Hz alternating current signal supplied through a 1 MΩ resistor to a constant-current source, in-phase voltage, and out-of-phase voltage were recorded with ECMS 1.0 software (CET, IA, USA). Endothelial barrier function was expressed as TER normalized to the baseline and the time when thrombin addition was set at 0. Only endothelial monolayers with a baseline TER of 5000 Ω or higher were used for experiments in this study.

2.5 Neutrophil transmigration
HUVECs (5 × 10^4) were seeded onto a 96-well transwell membrane (Millipore, CA, USA) and grown overnight to confluence. Human neutrophils were isolated from peripheral blood based on approval by the University of California at Davis Institutional Review Board and the investigation conforms to the Declaration of Helsinki. Neutrophils were separated with histopaque (Sigma, MO, USA) gradient centrifugation followed by negative selection (Stem Cell, BC, Canada) which yielded a purity >90%. 5 × 10^6 neutrophils were added to the top well with or without 10 nM fMLP (Sigma, MO, USA) in the bottom well. After a 2 h incubation, 5 × 10^4 polystyrene beads (Polysciences, PA, USA) were added to the bottom well and then migrated neutrophils were counted using FACs and normalized to 5000 beads. In parallel, the wells were submitted to MTT assay and Calcein AM staining to confirm cell quantity and confluence.

2.6 Conditioned medium analysis
Serum-free medium was collected and processed as described previously. The level of soluble VE-cadherin was detected using western blotting with an antibody against the extracellular domain of VE-cadherin (R&D, MN, USA).

2.7 Statistical analysis
For each intervention, multiple experiments were performed and data presented as mean ± SD. The number of samples (n) indicates the number of experiments done in separate days using different cells.
Two-tailed unpaired Student’s test was used for comparison between two groups and one-way analysis of variance used for multiple groups to evaluate the level of difference. Significance was accepted at $P \leq 0.05$.

3. Results

3.1 ADAM15 is a regulator of endothelial permeability

Since ADAM15 is upregulated in several inflammatory settings \(24-26\) that are associated with endothelial hyperpermeability, we aimed to determine the causal relationship between ADAM15 expression and endothelial permeability in HUVECs. We first verified that pcDNA/ADAM15 transfection increased ADAM15 expression by $\approx 60\%$ and siRNA/ADAM15 knockdown reduced its expression by $\approx 85\%$ (Figure 1A). Next, albumin permeability and electric resistance were measured in ADAM15-overexpressing or knockdown HUVECs. Thrombin, a permeability-increasing agonist contributing to vascular inflammation, \(28\) was used to stimulate the endothelial monolayers. In non-stimulated monolayers, ADAM15 overexpression resulted in a $35\%$ increase, whereas knockdown caused a rather small ($16\%$) decrease, in albumin permeability (Figure 1B), supporting the possibility that ADAM15 contributes to vascular barrier dysfunction under conditions where this molecule is upregulated. Furthermore, when thrombin was added, augmented hyperpermeability was observed in ADAM15-overexpressing cells (37% increase). Accordingly, ADAM15 knockdown caused a 20% decrease in $P_a$ in the presence of thrombin (Figure 1B), indicating a role for ADAM15 in mediating thrombin-induced hyperpermeability. Consistent with the albumin permeability assay, the TER dynamics showed a rapid drop in resistance (indicative of barrier dysfunction) upon thrombin. Overexpression of ADAM15 augmented the decrease in TER (maximal resistance $-0.39$ in mock-transfected control vs. $-0.48$ in ADAM15 overexpression) (Figure 1C). In contrast, ADAM15 knockdown attenuated thrombin-induced decreases in TER ($-0.40$ in scrambled siRNA control vs. $-0.26$ in siRNA ADAM15) (Figure 1C). These results demonstrate that ADAM15 overexpression increases endothelial permeability and it also contributes to thrombin-induced hyperpermeability.

3.2 ADAM15 does not cause VE-cadherin shedding

It has been shown that ADAM15 and ADAM10 shed E- and N-cadherins on the surface of epithelial and neuronal cells. \(18,29\) In endothelial cells, ADAM10 cleaves VE-cadherin thus facilitating T-cell transmigration. \(19\) These findings along with the suggestion that ADAM15 co-localizes with VE-cadherin \(30\) promoted us to test whether ADAM15 affected endothelial permeability by digesting VE-cadherin. We transfected cells with a cDNA mutant that produces dominant expression of a catalytically dead metalloproteinase. \(18\) Our data, however, suggested that the protease activity was not involved in VE-cadherin shedding or degradation. As shown in Figure 2A, expression of either WT or catalytically incompetent ADAM15 did not alter the level of soluble VE-cadherin in the extracellular medium (indicator of ectodomain shedding). Likewise, ADAM15 knockdown and overexpression produced the same level of soluble VE-cadherin. Further, there was no difference in the production of albumin.

\[ \text{Figure 1} \quad \text{ADAM15 regulates endothelial permeability. (A) Western blot analysis of ADAM15 expression in HUVECs non-tranfected (NT) or transfected with empty vector (mock), ADAM15 siRNA (siADAM15), scrambled siRNA (siScam), pcDNA/WT ADAM15 (ovrexp/WT). Top, representative blots; bottom, quantitative protein levels (n = 8). *Indicates } P < 0.05 \text{ vs. mock or NT. (B) Albumin permeability (Pa) measured by the ratio of transendothelial flux to concentration difference in ADAM15-overexpressing (ovrexp/WT) or knockdown (siADAM15) HUVECs in the absence (solid) or presence (open) of thrombin (25 nM, 60 min) (n = 3). *P < 0.05 vs. mock or NT (right) without thrombin; \#P < 0.05 vs. mock or NT with thrombin. (C) Transcellular electric resistance (TER) was measured in HUVECs as indicator of adhesive barrier function before and after thrombin (25 nM). Top, averaged peak response (n = 4). *P < 0.05 vs. mock or NT. Bottom, individual tracings showing the TER dynamic. Time 0 indicates the time when thrombin was added. } \]
the C-terminal fragment (CTF) of VE-cadherin in cells expressing WT and mutant ADAM15 or depleted of ADAM15 after treatment with the γ-secretase inhibitor DAPT which prevents secondary degradation and CTF removal (Figure 2B). Consistently, the functional assays showed that the expression of WT ADAM15 with intact protease function or ADAM15 mutant lacking protease activity increased albumin permeability and TER response to thrombin to a similar extent (Figure 2C), indicating that the protease activity is not required for ADAM15-induced hyperpermeability.

### 3.3 ADAM15 affects neutrophil transendothelial migration

We further determined the effect of endothelial ADAM15 on neutrophil transendothelial migration in the absence and presence of a chemoattractant, fMLP. In the absence of fMLP, expression of either WT or mutant ADAM15 in HUVECs increased neutrophil transmigration by two- to three-folds. In the presence of fMLP, WT and mutant ADAM15 caused a further increase in transmigration by more than 30% (Figure 3A). Knockdown of ADAM15 produced an opposite effect, reducing neutrophil transmigration with or without fMLP (Figure 3B).

### 3.4 ADAM15 activates ERK1/2 signalling independently of its protease activity

Because ERK1/2 phosphorylation is involved in permeability regulation by thrombin and other inflammatory mediators like histamine,32 we assessed whether ERK1/2 signalling contributed to the ADAM15 effect. In the absence of thrombin, overexpression of either WT or mutant ADAM15 increased ERK1/2 phosphorylation by nearly two-fold, whereas knockdown of ADAM15 decreased the

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**Figure 2** ADAM15-induced permeability is independent of its proteolytic effect on VE-cadherin shedding. (A) The level of soluble VE-cadherin measured by immunoblotting with an antibody against the extracellular domain of VE-cadherin in conditioned media. Full-length VE-cadherin (FL VE-cad) was measured in cell lysate as control. No difference detected in cells overexpressing ADAM15 wild-type (ovrexp/WT) and its mutant lacking protease function (ovrexp/mut) (n = 3). (B) Detection of VE-cadherin shedding products by immunoblotting with an antibody specific to the C-terminal fragment (CTF) of VE-cadherin in cells treated with the γ-secretase inhibitor DAPT. There is no difference in CTF production from cells expressing wild-type and catalytically dead ADAM15 (n = 3). (C) Comparison of ADAM15 level and permeability in HUVECs overexpressing ADAM15 wild-type (ovrexp/WT) and its mutant lacking protease function (ovrexp/mut). Left column shows consistent levels of ADAM15 expression regardless of mutation (n = 6). *P < 0.05 vs. mock cells. Middle column shows that ADAM15 WT or mutant increases albumin permeability and enhances thrombin-induced barrier dysfunction. *P < 0.05 vs. mock; #P < 0.05 vs. mock with thrombin (n = 3). Right column shows representative TER dynamics from three experiments.
phosphorylation by 30% (Figure 4A). When cells were stimulated with thrombin, overexpression (WT or mutant) further elevated ERK1/2 phosphorylation and knockdown produced an opposite effect (Figure 4A). The experiment using U0126, which blocks ERK1/2 activation, further supports the involvement of ERK1/2 signalling in ADAM15-mediated permeability. As shown in Figure 4B, while U0126 had no effect on the basal barrier properties of mock-transfected endothelial monolayers, it prevented ADAM15-induced increase in albumin permeability. Furthermore, U0126 pre-treatment inhibited thrombin-induced hyperpermeability; the inhibitory effect was more significant in ADAM15-overexpressing cells than in seen mock-transfected cells (Figure 4C). These data demonstrate an important role of ERK1/2 signalling in ADAM15 regulation of endothelial barrier function.

3.5 ADAM15-induced ERK1/2 activation requires Src but not focal adhesion kinase

ERK1/2 phosphorylation has been shown as a downstream event of focal adhesion kinase (FAK) or Src signalling. With an RGD sequence in its disintegrin domain, ADAM15 can bind αvβ3 and α5β1 integrins35 triggering outside-in signalling via FAK phosphorylation. We tested whether this pathway was responsible for ADAM15-induced ERK1/2 activation and barrier dysfunction. When adhered to collagen, fibronectin, or vitronectin (via different integrins), endothelial cells overexpressing WT or mutant ADAM15 displayed a slightly higher level of FAK phosphorylation at tyrosine 925 compared with mock-transfected cells (data not shown). However, depletion of FAK using siRNA had no effect on ADAM15-induced ERK1/2 activation (Figure 5A), indicating that FAK is not required for the response. In contrast, when Src activity was inhibited by PP2, a significant attenuation in ERK1/2 phosphorylation was observed (Figure 5B). Similar to U0126, PP2 was able to block the hyperpermeability induced by ADAM15 overexpression (Figure 5B).

Figure 3 ADAM15 promotes neutrophil transendothelial migration with and without chemoattractant. HUVECs expressing ADAM15 WT or mutant were grown on transwell membrane with neutrophils added to the top chamber and fMLP or vehicle added to the bottom. Transmigrated neutrophils were counted using FACS and normalized to the mock or non-treated condition. Top, transmigration across ADAM15 WT and mutant expressed HUVECs without (−) and with (+) fMLP (n = 3). Bottom, transmigration across ADAM15 knockdown and control siRNA treated HUVECs with or without fMLP (n = 3). *P < 0.05 vs. mock or NT cells without fMLP. **P < 0.05 vs. mock or NT with fMLP.

Figure 4 ADAM15 causes ERK1/2 phosphorylation coupled with hyperpermeability. (A) ERK1/2 phosphorylation (pERK1/2) was measured by blotting with anti-phospho ERK1/2 (T202/Y204) in HUVECs expressing ADAM15 WT or mutant in the absence and presence of thrombin (25 nM, 5 min). Total ERK1/2 (tErk1/2) was probed as control. Top, representative blots. Bottom, ratio of phosphorylated to total ERK1/2 (n = 5). *P < 0.05 vs. mock or NT without thrombin. **P < 0.05 vs. mock or NT with thrombin. (B) ERK1/2 phosphorylation (top) and albumin permeability (bottom) in ADAM15-overexpressing HUVECs during inhibition of ERK activation with U0126 (10 μM, 60 min). The inhibitor blocks ADAM15-induced increase in Pa (n = 3). *P < 0.05 vs. mock with vehicle; **P < 0.05 vs. ovrexp with vehicle. (C) ERK1/2 phosphorylation (top) and thrombin-induced TER changes (bottom) in ADAM15-overexpressing HUVECs with or without inhibition of ERK activation. ADAM15 overexpression enhances the TER decrease by thrombin and the effect is inhibited by UO126 (n = 3). *P < 0.05 vs. mock with vehicle; **P < 0.05 vs. ovrexp with vehicle.
3.6 Src and ERK1/2 are involved in ADAM15-facilitated neutrophil transmigration

Pre-treatment of endothelial cells with PP2 reduced neutrophil transmigration and the inhibitory effect was more significant in ADAM15 overexpressing cells than in mock-transfected cells (50 vs. 22% reduction). Pre-treatment of HUVECs with U0126 also attenuated the migration response but the effect was relatively modest (reduction by 10% in mock-transfected and 22% in ADAM15-expressing cells) (Figure 6).

4. Discussion

ADAM15 is upregulated in endothelial cells treated with pro-inflammatory cytokines and in tissues under various inflammatory settings, including atherosclerosis, rheumatoid arthritis, and irritable bowel.

In this study, we report that overexpression of ADAM15 in endothelial cells increases endothelial permeability and promotes neutrophil transmigration. Furthermore, ADAM15 enhances endothelial hyperpermeability to thrombin, and ADAM15 depletion attenuates thrombin-induced barrier dysfunction. The mechanism by which ADAM15 regulates permeability is not dependent on its proteolytic domain-mediated shedding of VE-cadherin or its disintegrin effect on cell–matrix focal adhesion; rather, it involves activation of Src and ERK1/2, perhaps via intracellular signalling. Our results have characterized ADAM15 as a novel endothelial barrier regulator and a potential therapeutic target of vascular inflammation.

This study indicates a role of ADAM15 in the pathological regulation of endothelial permeability in diseased vasculature where ADAM15 is upregulated or inflammatory mediators such as thrombin are elaborated. In particular, ADAM15 overexpression was capable of increasing albumin flux and augmenting thrombin-induced barrier dysfunction in endothelial cells. While alterations in the basal barrier property can contribute to enhanced or attenuated response to a stimulus, the effect of ADAM15 on resting endothelial cells was relatively modest compared with that seen with thrombin. In other words, altered basal permeability did not fully account for the altered thrombin response. Alternatively, ADAM15 may serve as a signalling molecule that mediates the permeability response under stimulated conditions. Such a condition is often seen in diseases associated with vascular inflammation or thrombosis where thrombin metabolism or signalling is altered. For example, in atherosclerosis, ADAM15 upregulation in the vascular endothelium may lead to endothelial hyperpermeability and augmented response to thrombin, contributing to the development of lesions. Further investigation with in vivo models of disease or genetically altered mice would provide physiologically significant information.

Based on the complex structure of the ADAM family, multiple molecular pathways have been proposed to explain their effects, of which the best recognized mechanism is proteolytic cleavage or ectodomain shedding of transmembrane molecules via its metalloproteinase domain. We first tested the hypothesis that ADAM15 induced hyperpermeability by shedding of VE-cadherin. Supporting this is that shedding of membrane-anchored VE-cadherin by metalloproteinases, measured as increased soluble fragments corresponding to the extracellular domain of VE-cadherin, is detected in vascular inflammatory disease. As a member of the metalloproteinase family, ADAM15 sheds E-cadherin on the epithelial cell surface generating soluble fragments that trigger ErbB activation. ADAM10, a close relative of ADAM15, cleaves VE-cadherin in association with endothelial permeability.

To our surprise, however, the data from the current study challenges the conventional concept that ADAMs act mainly through proteolysis. We found that ADAM15 with or without protease activity caused barrier responses to a similar extent, and they did not alter the level of soluble VE-cadherin or its degradation.
products, indicating that VE-cadherin shedding is not involved in ADAM15-induced permeability.

We then examined the potential importance of integrin-mediated focal adhesion in the ADAM15 effect. The extracellular part of ADAM15 contains an RGD sequence that binds to different integrins mediating heterotypic cell interactions. Overexpression of ADAM15 in epithelial cells facilitates leucocyte adhesion by mechanisms involving its RGD motif. Loss of ADAM15 in prostate tumour cells reduces their ability to adhere and migrate. In agreement with these studies, we found increased neutrophil transmigration across ADAM15-overexpressed endothelial cells. However, our results do not support the disintegrin effect (disruption of focal adhesion) as a critical step in the endothelial response to ADAM15, since ADAM15 overexpression did not alter integrin expression or cell attachment to different matrix proteins (data not shown). More importantly, siRNA depletion of FAK, a major mediator of integrin signalling, did not significantly alter ADAM15-induced endothelial responses, indicating that FAK is dispensable. Indeed, both RGD-dependent and -independent pathways have been proposed for ADAM15 action. An earlier study with NIH3T3 cells suggests that ADAM15 does not alter cell–matrix interactions but functions as a cell–cell adhesive molecule to decrease cell permeability. The discrepancy in the data on permeability is not surprising given the pleiotropic functions and heterogeneous effects of ADAM15 in different cells or tissues. Its effect on the endothelial barrier may not be the same as that seen with cells that do not form barriers or cells connecting to each other via other adhesion molecules than VE-cadherin, which is predominantly expressed in endothelial cell–cell junctions and serves as an important regulator of vascular permeability.

The negative finding about VE-cadherin shedding and focal adhesion disruption promoted us to explore the potential mechanism of intracellular signal transduction. ADAM15 possesses a cytoplasmic tail containing SH3/SH2 binding sequences and putative recognition sites for protein kinases capable of transducing complex signals or serving as molecular scaffold to recruit signalling components with kinase activity, such as Src, which in turn act on the endothelial barrier structure. In haematopoietic cells, the cytoplasmic tail of ADAM15 binds to Src in a phosphorylation-dependent manner. Mutation studies demonstrate the involvement of this cytoplasmic domain in homotypic aggregation of T cells as well as in heterotypic adhesion between lymphocytes and epithelial cells. Moreover, several isoforms of ADAM15 have been classified based on splicing variants of the cytoplasmic domain. One variant containing Src binding site with a high catalytic activity dependent on Src has been associated with malignant tumour behaviour. While these findings support in general the role of Src in the ADAM15 pathway, our data characterize the specific contribution of Src to endothelial responses to ADAM15. In another study, we found that truncation of its intracellular domain blocked ADAM15-induced increase in permeability, further supporting that intracellular signal transduction as an important mechanism underlying the endothelial effect of ADAM15.

ERK1/2 signalling has long been implicated in endothelial responses to growth factors and inflammatory mediators. In breast cancer cells, ADAM15 deletion decreases ERK1/2 phosphorylation impairing migration and proliferation. In this study, we demonstrate that ADAM15 overexpression stimulates ERK1/2 phosphorylation, an effect responsible for altered barrier function in the presence or absence of inflammatory stimulation. Furthermore, ADAM15-induced ERK1/2 activation is involved in neutrophil transendothelial migration, consistent with a previous observation. While ERK1/2 is known to be activated downstream from FAK and Src, we show that ADAM15-induced ERK1/2 activation is not dependent on FAK but largely through Src. This finding supports our hypothesis that the Src-binding cytoplasmic domain of ADAM15 serves as a triggering factor in the transduction of permeability responses. Further, additional signalling molecules may be involved in the ADAM15 pathway because Src inhibition did not completely block the phosphorylation response.

The precise mechanisms by which Src or ERK1/2 regulates permeability remain to be elucidated. We hypothesize that these kinases target the endothelial barrier structure for phosphorylation rendering weakened cell–cell adhesion. Our previous studies suggest that Src causes β-catenin tyrosine phosphorylation leading to intercellular gap formation, and that the ERK1/2 pathway mediates microvascular hyperpermeability by activating endothelial contractile cytoskeleton and altering cell–cell and cell–matrix interactions. A search of potential ERK targets using the consensus phosphorylation motif TXY produces several proteins that are known to be essential to endothelial barrier function, including FAK, VE-cadherin, catenins, actin, and actin-binding proteins. Although siRNA knockdown of FAK did not effectively abolish the permeability effect of ADAM15, we cannot rule out the possibility that post-translational modification of FAK activity or perturbation of integrin signalling contributes to the ADAM15 action. Some studies have shown a link between integrin activity and Src kinases. Likewise, while VE-cadherin shedding was not detected, it is possible that ADAM15 induce phosphorylation or conformational changes of the junction complex.

We also examined the effect of endothelial ADAM15 on neutrophil transendothelial migration because neutrophil diapedesis often occurs in parallel with endothelial barrier dysfunction during vascular inflammation. Neutrophils migrate across the endothelium via paracellular pathways maintained by junctional molecules including VE-cadherin, PECAM-1, and JAM-A. Despite the current focus on VE-cadherin, we recognize the role of PECAM-1 and JAM-A in regulating endothelial integrity and neutrophil transmigration and their potential involvement in the ADAM15 pathway. Additionally, the relative contribution of endothelial barrier properties vs. neutrophil activities to the transmigration process requires further studies. Within this context, the result that ADAM15 greatly increased neutrophil transmigration without a chemoattractant indicate the possibility that endothelial activation is sufficient to open or loosen the barrier for transmigration. Further stimulation or activation of neutrophils can augment the migration response but may not a prerequisite for the overall process. This may explain why the chemotactic migration to FMLP was not increased much more than the non-chemotactic migration. The data support an active role of endothelial ADAM15 in vascular inflammation.

In conclusion, our study has identified ADAM15 as a novel endothelial barrier regulator especially under inflammatory stimulation. The effects of ADAM15 on endothelial hyperpermeability and neutrophil transmigration are not dependent on the catalytic activity of the metalloproteinase but are mediated by intracellular signalling involving Src and ERK1/2 activation.

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