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CRISPR-mediated deletion of the PECAM-1 cytoplasmic domain increases receptor lateral mobility and strengthens endothelial cell junctional integrity

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Abstract

Aims—PECAM-1 is an abundant endothelial cell surface receptor that becomes highly enriched at endothelial cell-cell junctions, where it functions to mediate leukocyte transendothelial migration, sense changes in shear and flow, and maintain the vascular permeability barrier. Homophilic interactions mediated by the PECAM-1 extracellular domain are known to be required for PECAM-1 to perform these functions; however, much less is understood about the role of its cytoplasmic domain in these processes.

Main methods—CRISPR/Cas9 gene editing technology was employed to generate human endothelial cell lines that either lack PECAM-1 entirely, or express mutated PECAM-1 missing the majority of its cytoplasmic domain (CD-PECAM-1). The endothelial barrier function was evaluated by Electric Cell-substrate Impedance Sensing, and molecular mobility was assessed by fluorescence recovery after photobleaching.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.L. performed the majority of the studies, analyzed data, and wrote the manuscript. H.M. and Y.H. supplied funding, and helped edit the manuscript. D.K.N. designed experiments, analyzed data, and wrote the manuscript. P.J.N. designed experiments, analyzed data, and wrote the manuscript.

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Key findings—We found that CD-PECAM-1 concentrates normally at endothelial cell junctions, but has the unexpected property of conferring increased baseline barrier resistance, as well as a more rapid rate of recovery of vascular integrity following thrombin-induced disruption of the endothelial barrier. Fluorescence recovery after photobleaching analysis revealed that CD-PECAM-1 exhibits increased mobility within the plane of the plasma membrane, thus allowing it to redistribute more rapidly back to endothelial cell-cell borders to reform the vascular permeability barrier.

Significance—The PECAM-1 cytoplasmic domain plays a novel role in regulating the rate and extent of vascular permeability following thrombotic or inflammatory challenge.

Keywords

PECAM-1; glycosylation; sialic acid; endothelial cell; adhesion; permeability; vascular biology

INTRODUCTION

Platelet endothelial cell adhesion molecule (PECAM-1, CD31) is a 130-kDa member of the immunoglobulin (Ig) superfamily that is expressed on the surface of hematopoietic progenitor cells, leukocytes, and platelets, and is highly enriched at the intercellular junctions of confluent endothelial cell monolayers (Albelda et al., 1990, Muller et al., 1989, Newman et al., 1990). PECAM-1 is comprised of a 118-residue cytoplasmic domain, a 19-residue transmembrane domain, and an extracellular domain containing six Ig homology domains, the amino terminal two of which mediate PECAM-1/PECAM-1 homophilic interactions (Paddock et al., 2016, Sun et al., 1996a, Sun et al., 1996b). Extracellular domain-mediated homophilic binding is critical for concentrating PECAM-1 at endothelial cell-cell junctions (Sun et al., 2000, Wong et al., 2000), where it plays an important role in maintaining endothelial barrier integrity following thrombotic or inflammatory challenge – a function that has been demonstrated both in vivo (Carrithers et al., 2005, Ferrero et al., 1995, Graesser et al., 2002, Maas et al., 2005, Mahooti et al., 2000) and in vitro (Lertkiatmongkol et al., 2016, Mei et al., 2014, Privratsky et al., 2011).

The PECAM-1 cytoplasmic domain is encoded by eight exons (Kirschbaum et al., 1994), is largely unstructured (Paddock et al., 2011), and carries out multiple functions in endothelial cells. Specifically, it is required for PECAM-1 to (1) function as part of a mechanosensory complex (Collins et al., 2012, Conway and Schwartz, 2015, Kaufman et al., 2004, Tzima et al., 2005) (2) confer cytoprotection in response to proapoptotic stimuli (Bergom et al., 2006, Evans et al., 2001, Gao et al., 2003), and (3) interact with other junctional adhesion proteins and cytoskeletal molecules (Biswas et al., 2006, Biswas et al., 2003, Biswas et al., 2005, Ilan et al., 2000, Ilan et al., 1999, Tzima et al., 2005). Studies of fusion proteins that contain the PECAM-1 extracellular Ig domains, but transmembrane and cytoplasmic domains of ICAM-1, have demonstrated that the PECAM-1 cytoplasmic domain is not required for its border localization (Sun et al., 2000, Wong et al., 2000). Little is known, however, about the influence of the PECAM-1 cytoplasmic domain on barrier integrity in endothelial cells.

We employed CRISPR/Cas9 gene editing technology to generate a series of novel human endothelial cell lines that either lack PECAM-1 entirely, or express a mutant form of

PECAM-1 missing the majority of its cytoplasmic domain. These were then used to examine whether the PECAM-1 cytoplasmic domain regulates endothelial barrier function and, if so, how. Our results demonstrate that loss of the PECAM-1 cytoplasmic domain does not affect its ability to concentrate at the borders of confluent endothelial cells, but unexpectedly enhances its ability to maintain and restore endothelial junctional integrity after challenge. These results suggest that the ability of PECAM-1 to move freely within the plane of the plasma membrane is controlled by its cytoplasmic domain, which in turn determines the efficiency with which endothelial cells are able to establish and maintain their vascular permeability barrier.

RESULTS

Creation of PECAM-1-deficient and PECAM-1 cytoplasmic domain-deleted human immortalized endothelial cell lines

Previous studies examining the function of the PECAM-1 cytoplasmic domain have been carried out using murine NIH3T3 cells (Albelda et al., 1991, DeLisser et al., 1994), monkey Cos7 cells (Albelda et al., 1991), Chinese hamster ovary cells (Wong et al., 2000), murine L-cells (Albelda et al., 1991, Sun et al., 1996a), murine brain endothelioma cells (Tzima et al., 2005, Wong et al., 2000), bovine aortic endothelial cells (Tzima et al., 2005), and human mesothelioma cells (Sun et al., 2000). Although these cell lines grow as adherent monolayers that allow PECAM-1, via diffusion trapping, to concentrate at cell-cell junctions, potential cytoplasmic and/or plasma membrane partners likely vary widely between each of these cell lines and authentic human endothelial cells. Because such components may provide an important context for the function of the PECAM-1 cytoplasmic domain, we used CRISPR/Cas9 technology to edit the PECAM-1 gene in human endothelial cells *in situ* to produce two novel immortalized cell lines: one in which PECAM-1 is missing completely (KO-PECAM-1 iHUEVCs), and one in which only the PECAM-1 cytoplasmic domain has been deleted (CD-PECAM-1 iHUEVCs). A schematic diagram depicting sequences of the guide RNAs (gRNAs) used to create these cell lines, and the approximate location of their corresponding target sites in the PECAM-1 gene, is shown in Fig. 1. KO-PECAM-1 iHUEVCs were produced by transducing iHUEVCs with a lentiviral vector encoding the Cas9 nuclease and gRNA 1 (Fig. 1B) to create an insertion/deletion mutation resulting in a premature stop codon within PECAM-1 exon 1. CD-PECAM-1 iHUEVCs were created using a lentiviral vector encoding Cas9 and gRNAs 10 (Fig. 1C) and 16 (Fig. 1D), resulting in deletion of the cytoplasmic domain bounded by exons 10 through 16. The cysteine residue that becomes palmitoylated (Sardjono et al., 2006), as well as positively charged R and K residues that constitute the stop transfer sequence immediately inside the inner face of the plasma membrane, were intentionally left in place to prevent slippage of the transmembrane domain into and out of the lipid bilayer.

Deletion of the PECAM-1 cytoplasmic domain does not affect the ability of PECAM-1 to localize at endothelial cell-cell borders

Flow cytometry, employing monoclonal antibodies (mAbs) PECAM-1.3 and 235.1, which are specific for amino and C-termini of the PECAM-1, respectively (depicted in Fig 1.), was used to verify that KO-PECAM-1 iHUEVCs lacked PECAM-1 expression, while the CD-

PECAM-1 iHUECs expressed the extracellular, but not cytoplasmic, domain of PECAM-1. As expected, wild-type iHUECs bound both mAbs (Fig. 2A), CD-PECAM-1 bound only mAb PECAM-1.3 (Fig. 2B), while KO-PECAM-1 iHUECs bound neither (Fig. 2C). Confocal microscopy was then employed to assess the ability of wild-type PECAM-1 (Fig. 2D–F) and CD-PECAM-1 (Fig. 2G–I) to become concentrated at endothelial cell-cell junctions. Reconstruction of the Z-axis in each of these micrographs demonstrates that CD-PECAM-1 localizes to endothelial intercellular junctions to the same extent as does WT-PECAM-1, and both forms are largely absent from the apical surface in confluent endothelial cell monolayers.

The PECAM-1 cytoplasmic domain regulates baseline barrier function and the rate of restoration of endothelial cell junctional integrity following disruption by thrombin

Previous studies have shown the importance of PECAM-1 extracellular domain-mediated homophilic binding in the establishment and maintenance of the vascular barrier (Lertkietmongkol et al., 2016, Mei et al., 2014, Privratsky et al., 2011), but little is known about the contribution of the PECAM-1 cytoplasmic domain to endothelial cell barrier function. Electric Cell-substrate Impedance Sensing (ECIS) technology, which can monitor subtle changes in endothelial cell barrier function in real-time (Giaever and Keese, 1991), was used to determine whether the PECAM-1 cytoplasmic domain plays a role in regulating vascular permeability. iHUEC cell lines expressing WT-, CD-, or KO-PECAM-1 were plated on gold electrodes to form confluent monolayers, and thrombin was used to disrupt junctional integrity. As shown in Fig. 3, KO-PECAM-1 iHUECs had poorer baseline barrier resistance, and exhibited a significantly slower rate of recovery of endothelial cell barrier function following thrombin challenge, than did endothelial cells expressing wild-type PECAM-1, as expected. In contrast, CD-PECAM-1 iHUECs exhibited tighter baseline resistance and a faster rate and extent of recovery of barrier restoration, suggesting that the cytoplasmic domain of PECAM-1 regulates its ability to contribute to the endothelial cell permeability barrier.

Deletion of PECAM-1 cytoplasmic domain enhances the mobility of PECAM-1 within the plane of the endothelial cell plasma membrane

To account for the observation that cytoplasmic domain-deleted PECAM-1 forms tighter baseline barriers that are able to be restored more quickly following thrombotic or inflammatory challenge, we constructed lentiviruses encoding wild-type- and CD-PECAM-1 fused to green fluorescent protein (GFP), and transduced them into PECAM-1-deficient iHUECs. Flow cytometric and confocal microscopic analysis confirmed that both constructs were expressed to a similar degree on the cell surface (Fig. 4), and capable of concentrating at cell-cell borders (Fig. 4 **inset**). To examine the possibility that the cytoplasmic domain restricted the mobility of PECAM-1 within the plane of the endothelial cell plasma membrane, we subjected confluent monolayers of these cells to fluorescence recovery after photobleaching (FRAP) analysis. A high intensity laser was used to photobleach cell-cell junctional regions with similar intensities of PECAM-1 staining, after which the rate of recovery of fluorescence, which reports the migration of GFP-tagged molecules into the photobleached region, was quantified using confocal microscopy. As

shown in Figure 5, GFP- CD-PECAM-1 had a higher rate of diffusion within the plasma membrane compared to GFP-WT-PECAM-1.

DISCUSSION

PECAM-1 is a cell adhesion and signaling receptor expressed on the surface of platelets and leukocytes, and is also the most abundant cell surface molecule on endothelial cells. PECAM-1 becomes highly concentrated at endothelial cell junctions via diffusion trapping – a passive process in which PECAM-1 molecules, diffusing laterally within the lipid bilayer of the plasma membrane, come into contact with PECAM-1 molecules on adjacent cells, interact homophilically in *trans*, and become “trapped” at the cell junction, where they embellish the permeability barrier (Sun et al., 2000). While the presence of PECAM-1 at endothelial cell-cell borders has been known to contribute to junctional integrity for more than 20 years (Ferrero et al., 1995), and that its absence results in increased vascular permeability under conditions of inflammatory, mechanical, or thrombotic stress (Carrithers et al., 2005, Graesser et al., 2002, Maas et al., 2005, Mahooti et al., 2000), the precise mechanism by which PECAM-1 contributes to junctional integrity, both under steady-state conditions and following barrier disruption, is incompletely understood.

Recent studies examining the barrier properties of endothelial cells, and other validated model cell systems that express mutant forms of PECAM-1 have begun to shed light on the molecular requirements for PECAM-1 to contribute to endothelial cell junctional integrity. Privratsky et al. knocked down expression of PECAM-1 in three different endothelial cell lines using siRNAs, and found, using ECIS technology, that loss of PECAM-1 resulted in both markedly reduced baseline barrier resistance, as well as a significantly decreased capacity to re-establish the endothelial cell permeability barrier following thrombin challenge (Privratsky et al., 2011). Cells expressing a mutant form of PECAM-1 containing a single K89A mutation in Ig domain 1 known to abolish homophilic binding (Newton et al., 1997) exhibited poor barrier resistance properties, similar to those of cells expressing no PECAM-1 at all, demonstrating the importance of PECAM-1-mediated homophilic binding to junctional integrity. Lertkiatmongkol et al. have recently shown that a sialic acid-containing glycan emanating from N25 reinforces dynamic endothelial cell-cell interactions by stabilizing the PECAM-1 homophilic binding interface (Lertkiatmongkol et al., 2016).

The importance of the PECAM-1 cytoplasmic domain in PECAM-1-mediated barrier functions has been less well studied. Cells expressing a mutant form of PECAM-1 in which functionally important immunoreceptor tyrosine-based inhibitory motif (ITIM) tyrosine residues had been mutated to phenylalanine exhibited normal baseline barrier resistance and restored vascular barrier integrity at a rate indistinguishable from cell expressing wild-type PECAM-1 (Privratsky et al., 2011), demonstrating that ITIM-mediated cellular signaling downstream of PECAM-1-mediated homophilic binding plays no discernable role in supporting the endothelial cell permeability barrier. Whether other structural or functional features of the cytoplasmic domain might play a role in PECAM-1-mediated contributions to barrier function, however, were not examined.

In the present investigation, we employed CRISPR/Cas9 gene editing technology to remove exons 10–16 of the PECAM-1 gene in human endothelial cells (Fig. 1) so that we could examine the effects of deleting the cytoplasmic domain on both baseline and dynamic cell permeability barrier function. Editing the PECAM-1 gene *in situ* has the advantage of not affecting gene expression levels (Fig. 2A–C), while retaining the cellular and molecular regulatory context in which PECAM-1 is normally expressed. Consistent with previous studies employing cell lines transfected with cDNAs encoding PECAM-1 isoforms either lacking the cytoplasmic domain (DeLisser et al., 1994) or containing an irrelevant cytoplasmic domain (Sun et al., 2000, Wong et al., 2000), absence of the PECAM-1 cytoplasmic domain had no effect on the ability of PECAM-1 to, with time, localize to cell-cell borders (Fig. 2D–I and Fig. 4 insets). When the kinetics of PECAM-1 receptor mobility were quantitatively examined using FRAP analysis of GFP-tagged forms of PECAM-1 with or without a cytoplasmic tail, however, we found that PECAM-1 receptors missing their cytoplasmic domain diffused more rapidly within the plane of the plasma membrane (Fig. 5) – a property that manifests itself functionally by conferring both improved baseline barrier resistance and a faster rate of re-establishing a permeability barrier following its disruption by thrombin (Fig. 3). Taken together, these data suggest that the PECAM-1 cytoplasmic domain, perhaps via ITIM-independent interactions with one or more as yet unidentified cytosolic binding partners, functions as a previously unrecognized point of regulation by restraining PECAM-1 receptor mobility within the plane of the plasma membrane and the subsequent homophilic interactions that are important for forming the endothelial cell permeability barrier.

Cytoplasmic domains have been shown to regulate the function and subcellular location of many cell adhesion molecules, including vascular endothelial cadherin (Kowalczyk et al., 1998), integrins (Chan et al., 1992, Marcantonio et al., 1990, O'Toole et al., 1991), selectins (Kansas et al., 1993), CD44 (Lesley et al., 1992), as well as other Ig superfamily CAMs (Carpen et al., 1992, Powell et al., 1991, Shin et al., 1991), often via their association with one or more elements of the actin or membrane cytoskeleton. The removal of the β -catenin-binding domain within the cytoplasmic tail of VE-cadherin has been reported to result in disorganization of adherens junctions and hyperpermeability of vascular endothelial cells (Miyazaki et al., 2011). Similarly, calpain-mediated cleavage of N-cadherin showed reduced cell-cell adhesion (Jang et al., 2009), while calpain cleavage of E-cadherin is involved in tumor progression (Trillsch et al., 2016, Ye et al., 2013). Thus, deletion of the cytoplasmic domain of cadherins results in distinctly different cell biological effects than does deletion of the PECAM-1 cytoplasmic domain. Su et al has shown that the decreased barrier function could be attributed to the increased degradation of VE-cadherin after the truncation (Su and Kowalczyk, 2017). Although, as important components at intercellular junctions, PECAM-1 and cadherins share some similarities in terms of structure and functions, cadherins and PECAM-1 are quite distinct in their lateral border localization and detergent extractability (Ayalon et al., 1994), reflecting differences in their mode of association with the cytoskeleton. As for PECAM-1, Ayalon et al. reported more than 20 years ago that a proportion of PECAM-1 is associated with the Triton-insoluble cytoskeleton in endothelial cells (Ayalon et al., 1994), and there are numerous reports that PECAM-1 may be linked to the cytoskeletal adapter molecules, β and γ catenin (Biswas et al., 2006, Biswas et al., 2003,

Biswas et al., 2005, Ilan et al., 2000, Ilan et al., 1999), though this has not been universally observed (Lampugnani et al., 1995). Wong et al. reported that PECAM-1 can be associated in *cis* with the integrin $\alpha v\beta 3$ (Wong et al., 2000), which is in turn associated with the actin cytoskeleton. In stimulated platelets, PECAM-1 may be linked to the actin cytoskeleton through the cytosolic adaptor protein, moesin (Gamulescu et al., 2003), and in endothelial cells under conditions of fluid shear stress with the intermediate filament protein vimentin (Conway et al., 2013). Whether these or other cytoskeletal proteins associate with PECAM-1 to restrict its mobility under resting or shear conditions, or in response to endothelial cell injury should be a fascinating topic for future investigations.

CONCLUSIONS

These results demonstrate that, in the absence of its cytoplasmic domain, PECAM-1 is freer to diffuse within the plane of the plasma membrane, migrate to, and become concentrated at endothelial cell-cell junctions, where it engages in homophilic interactions that establish and maintain barrier function. Whether other mechanisms may account for regulation of endothelial barrier function by the truncation of PECAM-1's cytoplasmic domain remains an intriguing question, the elucidation of which will have important implications for understanding the pathological processes in vascular permeability disorders.

MATERIALS AND METHODS

Antibodies

Domain-specific mouse anti-human PECAM-1 monoclonal antibodies (mAbs) used in this study include: 235.1 (specific for the C-terminal 15 amino acids), and PECAM-1.3 (specific for Ig Domain 1), and have been previously described (Newman et al., 2002, Yan et al., 1995). Normal mouse IgG and secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA). Alexa Fluor® 647-labeled mAb PECAM-1.3 and Alexa Fluor® 647-labeled mAb 235.1 were generated using a labeling kit purchased from Thermo Fisher Scientific (Waltham, MA).

Guide RNA plasmid constructs

Guide RNAs (gRNAs) were designed using the clustered regularly interspaced short palindromic repeats (CRISPR) Design Tool (<http://crispr.mit.edu/>) to minimize off-target effects and selected to precede a 5'-NGG protospacer-adjacent motif (PAM). gRNAs used in this study were: gRNA1 forward: 5'-CACCGCAGGACTCCAAGCCACATCG-3', reverse: 5'-AAACCGATGTGGCTT-GGAGTCCTGC-3'; gRNA10 forward: 5'-CACCGAGATGCCAGTGGAAATGTCC-3', reverse: 5'-AAACGGACATTTCCACTGGCATCTC-3'; gRNA16 forward: 5'-CACCGTTGTCGCTAC-AGAGAACGGA-3', reverse: 5'-AAACTCCGTTCTCTGTAGCGACAAC-3'. Oligos were annealed and cloned into the BsmBI site of the Cas9 expression plasmid lentiCRISPR v2 (#52961, Addgene, Cambridge, MA) following a previously described protocol (Sanjana et al., 2014).

Cell lines and transduction

Cell culture reagents were obtained from Mediatech (Manassas, VA) unless otherwise specified. Immortalized human umbilical vein endothelial cells (iHUEC, generated by transducing HUVECs with the recombinant retrovirus LXSNI6 E6/E7) and PEC02 cells (generated by transducing iHUECs with a lentivirus expressing a PECAM1-specific siRNA PEC02 - (Privratsky et al., 2011)) were maintained in RPMI1640 medium (Mediatech), 10% FBS (Sigma, St Louis, MO), 5% human AB serum (Gemini, West Sacramento, CA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 mg/ml endothelial cell growth supplement (Corning, Corning, NY) as previously described (Privratsky et al., 2011).

Generation of a PECAM-1-deficient immortalized HUVEC line

Lentiviruses were generated and titrated by the Viral Vector Core Facility at the Blood Research Institute. iHUECs cells were plated on 24-well plates one day before transduction, and transduced with gRNA1 lentiviral particles at an MOI of 5 in iHUECs media containing 0.8 µg/ml polybrene. After 48 hours, media containing puromycin (0.5 µg/ml) was added. Transduced cells were stained with 30 µg/ml Alexa Fluor 647-labeled mAb PECAM-1.3 and sorted by flow cytometry (ARIA-IIIu Cell Sorter, BD Biosciences, San Jose, CA) for PECAM-1 negative cells. KO-PECAM-1 endothelial cells were maintained in iHUEC medium containing puromycin.

Generation of CD-PECAM-1 iHUEC cell line

iHUECs were plated on 24-well plates one day before transduction, and then transduced with a 1:1 mixture of gRNA10 and gRNA16 lentiviral particles at an MOI of 5 in iHUECs media containing 0.8 µg/ml polybrene. Forty-eight-hours post-transduction, puromycin (0.5 µg/ml) was added to the medium. Cells were sorted as single cells into individual wells of 96-well plates 15 to 18 days post-puromycin selection. HUVECs in which PECAM-1 had been knocked down using an siRNA (PEC02 cells (Privratsky et al., 2011)) were used as feeder cells. Three weeks later, clones were stained by 30 µg/ml Alexa Fluor 647-labeled mAb PECAM-1.3 and sorted by flow cytometry to obtain a PECAM-1-positive population, and puromycin was introduced into the medium for 48 hours to further remove the feeder cells.

Characterization of endothelial cell lines by flow cytometry

Flow cytometric analysis of WT-, CD-, or KO-PECAM-1 iHUECs was performed using a BD Cytofix/Cytoperm™ (BD Biosciences, San Jose, CA) according to the manufacturer's directions. Briefly, Alexa Fluor® 647-labeled mAb PECAM-1.3 (30 µg/ml) was used in surface staining, and Alexa Fluor® 488-labeled mAb 235.1 (30 µg/ml) was used in intracellular staining. Flow cytometry was performed using a Becton-Dickenson LSRII (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Confocal microscopy

Cells were plated at a density of 2×10^5 /well on gelatin-coated slide chambers (BD Biosciences) at least 24 hours before staining. Wells were rinsed with DPBS, cells fixed with 2% paraformaldehyde for 20 minutes, and then permeabilized with ice-cold 0.5% Triton X-100 for 2 minutes, and blocked with PBS containing 3% BSA. Monolayers were then incubated with mAb PECAM-1.1 (10 μ g/ml) at room temperature for 1 hour. Binding was detected using Alexa Fluor 647 anti-mouse IgG (Invitrogen) and images were obtained using a FluoView FV1000 multi-photon emission microscope (Olympus, Center Valley, PA). Cross sectional reconstruction was accomplished with MetaMorph (Molecular Devices, Inc., Nashville, TN) workstation to combine the entire Z series into a stacked projection.

ECIS measurements of endothelial barrier function

Cells were grown to confluence on gold electrodes that had been coated with 0.1% gelatin (Invitrogen, Carlsbad, CA) for an hour at 37°C and subjected to Electric Cell-substrate Impedance Sensing (ECIS) analysis using an ECIS Z-Theta Instrument (Applied Biophysics, Troy, NY). To measure PECAM-1-mediated endothelial cell barrier function, cells were grown in 400 μ l of iHUEC medium on 8W10E+ electrode arrays until forming a tight monolayer. After stimulating cells with 1 unit of human thrombin (Sigma-Aldrich, St. Louis, MO), endothelial barrier disruption and restoration were measured in real time and continuously recorded at multiple frequencies and modeled with ECIS software (Applied Biophysics, Troy, NY) to obtain the barrier function parameter, Rb, which is expressed as the average basal electrical resistances (in Ωcm^2).

Generation and characterization of endothelial cell lines expressing GFP-PECAM-1 chimeric proteins

A silent mutation was introduced into the PAM sequence corresponding to guide 1 in PECAM-1 cDNA using QuikChange II site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) in order to protect it from CRISPR/Cas9 using the following oligonucleotide primers: (1) Fwd, 5'-GGTGGGCCCCAAGGGGCGACCATGTGGCTTGGAGTC-3'; (2) Rev, 5'-GACTCCAAGC-CACATGTCGCCCCCTGGGCCCCACC-3' (silent mutation sites are underlined). Mutated full-length human PECAM-1 or CD PECAM-1 was inserted into the backbone plasmid pLenti CMV GFP Neo (Addgene # 17447) using an In-Fusion® HD Cloning Kit (Clontech, Laboratories, Inc., Mountain View, CA) to generate a GFP-PECAM-1 chimeric protein. A linker sequence was inserted between GFP and the mutated WT-PECAM-1 or CD-PECAM-1. The primers for GFP-WT-PE chimeric protein were: forward, 5'-GGTGGCGGAGGCTCTCAAGAAAACCTTTTCA-CAATCAACAG-TGTT-3'; reverse, 5'-GAGGTTGATTGTGCGACCTAAGTTCCATCAAGGG-AGCCTT-3'. The primers for GFP-CD-PECAM-1 chimeric protein were: forward, 5'-GGTGG-CGGAGGCTCTCAAGAAAACCTTTTCAATCAACAGTGTT-3'; reverse, 5'-GAGGTTG-ATTGTGCGACCTACTTGGCCTTGGCTTTCCTCA-3'. KO-PECAM-1 cells were seeded in 24-well plates and transduced with lentiviral particles containing GFP-WT-PECAM-1 or GFP-CD-PECAM-1 at an MOI of 5 in 0.8 μ g/ml polybrene-containing iHUEC media. Transduced cells were further selected by addition of 0.5 mg/ml G418 48

hours post-transduction. Cell lines were sorted for Alexa Fluor 647-labeled mAb PECAM-1.3-positive expression.

Fluorescence Recovery after Photobleaching (FRAP)

Cells were plated on a 60 mm-diameter dish (Celltreat, Pepperell, MA) and cultured for at least 24 hours until cell monolayers had reached confluence. FRAP experiments were designed and performed as previously described (Day et al., 2012) using an FV1000 laser-scanning confocal microscope (Olympus, Tokyo, Japan) with a 100X objective lens. GFP fluorescence was imaged by excitation with a 488-nm laser at 37°C with 5% CO₂ using an Air-Therm-H heater (World Precision Instrument, Sarasota, FL). The region of interest at cell-cell contacts was selected and bleached for 300 ms using a 405 nm laser. Fluorescence intensity was monitored continuously and images were acquired until recovery reached a plateau using the FluoView-ASW-10 version 04.02.02.09 software (Olympus, Tokyo, Japan). The fluorescence intensity of the bleached region was corrected for photobleaching and normalized to prebleaching fluorescence intensity by ImageJ (NIH, Bethesda, MD). This algorithm allows computation of the diffusion coefficient of the fluorescent probes (2D random walk). Prism 5 software (GraphPad Software, San Diego, CA) was used for plotting the data and statistical analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Data were analyzed by one-way ANOVA followed by Holm-Sidak's multiple-comparisons test. Multiple comparisons tests were only applied when a significant difference was determined in the ANOVA ($p < 0.05$). Results are expressed as mean $\pm$ S.D.

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Figure 1. Strategy used to generate PECAM-1 knockout and cytoplasmic domain-deleted iHUVeC cell lines

(A) Schematic of PECAM-1 showing the locations of antibody binding sites for mAb PECAM-1.3, specific for PECAM-1 IgD1, and mAb 235.1, specific for the C-terminus of the PECAM-1 cytoplasmic domain. (B) Guide RNA (gRNA) sequence (orange bar) and the protospacer adjacent motif (PAM) sequences (blue) used to introduce an insertion/deletion in exon 1 of the PECAM-1 gene to generate a PECAM-1-deficient iHUVeC line (KO-PECAM-1). (C–D) Sequence of the gRNAs that frame the PECAM-1 cytoplasmic domain used to generate an iHUVeC line expressing PECAM-1 lacking its cytoplasmic domain (CD-PECAM-1). The approximate location of the binding sites of the gRNA relative to their location in exons 1, 10 and 16 are shown schematically in orange in panel A.

Figure 2. Characterization of CRISPR-generated iHUEC cell lines

Flow cytometric data showing the binding of mAbs PECAM-1.3 and 235.1 to wild-type iHUECs (**panel A**), Δ CD-PECAM-1 iHUECs (**panel B**), and knockout PECAM-1 iHUECs (**panel C**). Note the comparable surface expression levels of PECAM-1 in the WT and Δ CD iHUEC cell lines, but absence of cytoplasmic tail in the Δ CD iHUEC line. (**D–I**) Confocal fluorescence microscopy showing combined projection images (**Panels D and G**), as well as representative cross-sectional images (denoted by white lines) of representative z-planes (**Panels E, F, H, and I**) in iHUEC cells expressing either WT-PECAM-1 or Δ CD-PECAM-1. Note that absence of the PECAM-1 cytoplasmic domain does not affect its ability to concentrate at endothelial cell-cell borders. Scale bar = 20 μ m.

Figure 3. Absence of the PECAM-1 cytoplasmic domain confers enhanced baseline barrier function and faster restoration of endothelial cell junctional integrity following thrombin challenge

(A) ECIS analysis of the endothelial cell permeability barrier under resting and stimulated conditions of iHUECs expressing WT and Δ CD forms of PECAM-1. The lines display the mean $\pm$ s.d. of the resistance (Ω) over time. The dashed box indicates the time frame used to calculate the rate of recovery. N=10 for the WT and KO-PECAM-1 iHUEC lines, and 15 for the Δ CD-PECAM-1 cell line. (B) Modeled barrier function (Rb) of data shown in panel A. (C) Linear regression analysis of the resistance curves showing the mean $\pm$ s.d. of the slope from the nadir immediately after thrombin challenge to a point near full recovery. Statistics were carried out using one-way ANOVA analysis. *P<0.05; **P<0.01; ***P<0.001. Note the increased baseline barrier resistance as well as the enhanced rate of barrier recovery following thrombin stimulation of iHUECs expressing Δ CD-PECAM-1.

Figure 4. Generation of iHUEC cell lines expressing full-length and Δ CD forms of human PECAM-1 fused to GFP

Lentiviral constructs encoding fusion proteins comprised of full-length wild-type PECAM-1 fused to GFP, or Δ CD-PECAM-1 fused to GFP, were transduced into CRISPR-generated PECAM-1-negative iHUEC cells. Transduced cells expressing similar levels of PECAM-1 were selected by fluorescence-activated cell sorting, and expression additionally evaluated by confocal microscopy (insets).

Figure 5. Fluorescence recovery after photobleaching (FRAP) analysis of the lateral mobility of PECAM-1 within the plane of the plasma membrane

GFP-positive cells that had formed well-defined cell-cell junctions were subjected to FRAP analysis as described in Materials and Methods. Representative images of iHUVeCs expressing wild-type (**panel A**) and Δ CD-PECAM-1 (**panel B**) fused to GFP are shown at the indicated time points before and after laser-induced photobleaching. Photobleached areas are marked by white circles. (**C**) Normalized fluorescence intensity of GFP-WT-PECAM-1 (red) and GFP- Δ CD-PECAM-1 (blue) in the photobleached areas over time following photobleaching. (**D**) The diffusion coefficients for wild-type and Δ CD-PECAM-1 were calculated from the FRAP images using ImageJ. Data are expressed as the mean $\pm$ the standard deviation of seven independent experiments. Significant differences are indicated as ** $P < 0.01$. Scale bar = 20 μ m.