

Relative contribution of PECAM-1 adhesion and signaling to the maintenance of vascular integrity

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Summary

PECAM-1 (CD31) is a cellular adhesion and signaling receptor that is highly expressed at endothelial cell–cell junctions in confluent vascular beds. Previous studies have implicated PECAM-1 in the maintenance of vascular barrier integrity; however, the mechanisms behind PECAM-1-mediated barrier protection are still poorly understood. The goal of the present study, therefore, was to examine the pertinent biological properties of PECAM-1 (i.e. adhesion and/or signaling) that allow it to support barrier integrity. We found that, compared with PECAM-1-deficient endothelial cells, PECAM-1-expressing endothelial cell monolayers exhibit increased steady-state barrier function, as well as more rapid restoration of barrier integrity following thrombin-induced perturbation of the endothelial cell monolayer. The majority of PECAM-1-mediated barrier protection was found to be due to the ability of PECAM-1 to interact homophilically and become localized to cell–cell junctions, because a homophilic binding-crippled mutant form of PECAM-1 was unable to support efficient barrier function when re-expressed in cells. By contrast, cells expressing PECAM-1 variants lacking residues known to be involved in PECAM-1-mediated signal transduction exhibited normal to near-normal barrier integrity. Taken together, these studies suggest that PECAM-1–PECAM-1 homophilic interactions are more important than its signaling function for maintaining the integrity of endothelial cell junctions.

Key words: PECAM-1, CD31, Vascular permeability, Adhesion molecules

Introduction

Platelet endothelial cell adhesion molecule (PECAM)-1 (CD31) is a 130 kDa type I transmembrane glycoprotein that is expressed on most cells of the hematopoietic lineage including platelets, monocytes, neutrophils and certain lymphocyte subsets (Newman, 1997; Newman, 1999; Newman and Newman, 2003). Endothelial cells also express PECAM-1, and at 1×10^6 to 2×10^6 copies/cell (Newman, 1994). PECAM-1 is a primary constituent of endothelial cell–cell junctions in confluent vascular beds (Albelda et al., 1990; Muller et al., 1989; Newman et al., 1990; Newman, 1994), where it has been shown to have an important role in supporting transendothelial migration of leukocytes (Muller et al., 1993; O'Brien et al., 2003; Vaporciyan et al., 1993; Wakelin et al., 1996). The extracellular domain of PECAM-1 is comprised of six Ig-like homology domains, followed by a 19-residue transmembrane domain and a 118-residue cytoplasmic tail (Newman et al., 1990). N-terminal extracellular Ig domain-1 contains residues that are important for mediating homophilic PECAM-1–PECAM-1 interactions that direct PECAM-1 to cell–cell junctions (Bergom et al., 2008; Newton et al., 1997; Sun et al., 2000; Sun et al., 1996), whereas Cys595, which lies immediately inside the plasma membrane, has been shown to become post-translationally palmitoylated and targets PECAM-1 to membrane microdomains, where it regulates both cell signaling and apoptosis (Gratzinger et al., 2003; Lee et al., 2006; Sardjono et al., 2006). The PECAM-1

cytoplasmic domain contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) centered around Tyr663 and Tyr686 that become phosphorylated in response to a variety of cellular activation events, after which they are able to recruit a number of Src homology 2 (SH2) domain-containing cytosolic signaling molecules, the best known of which is the protein-tyrosine phosphatase SHP-2 (Newman and Newman, 2003). Other SH2 domain-containing proteins that have been reported to associate with PECAM-1 include SHP-1 (Cao et al., 1998; Henshall et al., 2001; Hua et al., 1998; Pumphrey et al., 1999; Sagawa et al., 1997), Src family kinases (Lu et al., 1997; Masuda et al., 1997; Osawa et al., 1997), the inositol 5'-phosphatase SHIP-1 (Pumphrey et al., 1999) and phospholipase C γ 1 (Henshall et al., 2001; Pumphrey et al., 1999).

Breach of vascular integrity results in the accumulation of plasma, proteins and cells in the interstitial space, and is one of the cardinal signs of the inflammatory response (Kumar et al., 2004). Tight regulation of the vascular permeability barrier is required to hold both acute and chronic inflammatory disease in check, and failure to restore barrier function in a timely manner can result in a catastrophic loss of vascular volume, as in septic shock (Cohen, 2002), or contribute to the development of chronic inflammatory diseases such as atherosclerosis (Sima et al., 2009; Vandenbroucke et al., 2008). Vascular permeability is regulated by the coordinated opening and closing of endothelial cell–cell junctions and relies on

a complex interplay of junctional adhesion components, cytoskeletal rearrangements, and cellular adhesive and counter-adhesive forces (Mehta and Malik, 2006). Consequently, molecules or proteins that hasten these processes, or that strengthen adhesive bonds between cells or the extracellular matrix, have the potential to prevent the increase in permeability that ultimately leads to tissue and organ dysfunction.

Ferrero and colleagues reported more than 15 years ago that PECAM-1-specific F(ab')₂ antibody fragments augment transit of ¹²⁵I-labelled albumin across endothelial cell junctions, both in cultured cells and in mice (Ferrero et al., 1995). Conversely, they also found that cultured NIH/3T3 and ECV304 cells could be made less permeable to albumin if the cells were transfected with cDNA encoding PECAM-1, and were the first to suggest that PECAM-1 contributes to endothelial cell barrier function. It was therefore somewhat surprising to discover that vascular development and function is overtly normal in PECAM-1-deficient mice (Duncan et al., 1999). However, a number of studies have since shown that the stability of endothelial cell junctions is more easily compromised in the blood vessels of PECAM-1-deficient mice subjected to physiological stress. For example, tail vein bleeding times are prolonged in PECAM-1-deficient versus wild-type (WT) mice; a phenotype that segregates with the vascular endothelium rather than with circulating platelets (Mahooti et al., 2000). Further support for a role for PECAM-1 in the barrier function of endothelial cells *in vivo* was provided by the studies of Graesser and colleagues (Graesser et al., 2002), who observed that re-establishment of a vascular permeability barrier is delayed in the vessels of PECAM-1-deficient mice in skin exposed to histamine, and in the brain microvasculature of mice suffering from experimental autoimmune encephalomyelitis. The most dramatic consequence of the absence of PECAM-1 from endothelial cell junctions, however, becomes evident during septicemia: two different groups have found that PECAM-1-deficient mice are much more susceptible to lipopolysaccharide (LPS)-induced septic shock than are their wild-type counterparts (Carrithers et al., 2005; Maas et al., 2005). When challenged with LPS, the blood vessels of PECAM-1-deficient mice exhibit increased permeability, and have an exaggerated loss of blood volume with a concomitant fatal drop in blood pressure. Similarly to the bleeding time phenotype described above, this could be corrected by expressing PECAM-1 solely on endothelial cells. Taken together, these data support the notion that PECAM-1-mediated adhesion and/or signaling contributes appreciably to vascular integrity and maintenance of a stable vascular permeability barrier, especially following disrupting stimuli.

Despite compelling evidence for a role for PECAM-1 as a prominent functional component of the endothelial cell–cell junction, the specific mechanism by which PECAM-1 functions to maintain or repair vascular barrier integrity is still poorly understood. The observation that PECAM-1 becomes highly concentrated at endothelial cell–cell junctions via diffusion trapping (Sun et al., 2000) suggests that PECAM-1–PECAM-1 homophilic interactions might be required for PECAM-1-mediated barrier protection. Alternatively, signaling mediated by PECAM-1 cytoplasmic ITIMs has been suggested to have a role in maintaining the vascular barrier because PECAM-1 expression has been reported to modulate β -catenin phosphorylation and enhance vascular barrier stability through ITIM-mediated recruitment of SHP-2 (Biswas et al., 2006). Two other groups, however, found that ITIM-mediated recruitment of SHP-2 by

endothelial cell PECAM-1 is not required for leukocyte transmigration through cell monolayers (Dasgupta et al., 2009; O'Brien et al., 2003), a process that involves the coordinated opening and closing of cell–cell junctions, which is very similar to the process occurring during regulation of vascular permeability. Finally, a role for raft-localized PECAM-1 is suggested by a report that PECAM-1 modulates signaling from the sphingosine-1-phosphate (S1P) receptor (Gratzinger et al., 2003) – a G-protein-coupled receptor that promotes barrier protection by enhancing junctional assembly via signaling in lipid rafts (Komarova et al., 2007).

Because it is currently not known which, if any, of these properties are important to enable PECAM-1 to contribute to endothelial cell junction stability, we sought to identify the pertinent biological properties of PECAM-1 (i.e. homophilic adhesion, localization to membrane microdomains, ITIM-mediated signaling) that allow it to establish and maintain vascular integrity. Using electric cell-substrate impedance sensing (ECIS) technology as a measure of endothelial cell junctional integrity, we found that endothelial cell monolayers expressing PECAM-1 exhibited significantly increased resistance to current flow at rest, as well as more rapid restoration of barrier integrity following thrombin-induced barrier disruption when compared with values for PECAM-1-deficient cells. In addition, cells that expressed a homophilic binding-crippled mutant form of PECAM-1 were unable to efficiently establish or restore the vascular barrier, whereas cells expressing variant forms of PECAM-1 lacking key residues known to be involved in PECAM-1-mediated signal transduction and lipid raft localization exhibited normal to near-normal barrier function, both at rest and following thrombin stimulation. Taken together, these results suggest that the ability of PECAM-1 to engage homophilically and localize to endothelial cell–cell borders is more important than its signaling function in conferring vascular barrier protection.

Results

PECAM-1 contributes to endothelial cell–cell junctional integrity

Previous *in vitro* and *in vivo* studies have shown that PECAM-1 promotes vascular barrier integrity (Biswas et al., 2006; Carrithers et al., 2005; Ferrero et al., 1995; Graesser et al., 2002; Maas et al., 2005). The biological mechanisms behind PECAM-1-mediated barrier protection, however, are still poorly understood. To confirm a role for PECAM-1 in both the steady-state maintenance of endothelial cell barrier function, as well in restoration of vascular integrity following its perturbation, we used ECIS technology as a measure of barrier integrity and cell–cell interactions because of its ability to quantitatively measure changes in monolayer resistance and cell micromotion in real time at the nanometer level (Giaever and Keese, 1991; Tirupathi et al., 1992). In addition, through the use of established formulas, ECIS is able to model the amount of current that is passing between cells (reported as the derived parameter *R_b*, resistance of the barrier), which is a robust reporter of barrier function (see the Materials and Methods) (Giaever and Keese, 1991). Primary human pulmonary artery endothelial cells (HPAECs) and human aortic endothelial cells (HAECs) were transiently transfected with pools of control or PECAM-1-specific siRNA oligonucleotides to silence endogenous levels of PECAM-1 (Fig. 1A,B), and then subjected to ECIS measurements. As shown in Fig. 1D,E, PECAM-1-expressing endothelial monolayers exhibited significantly increased steady-state barrier function

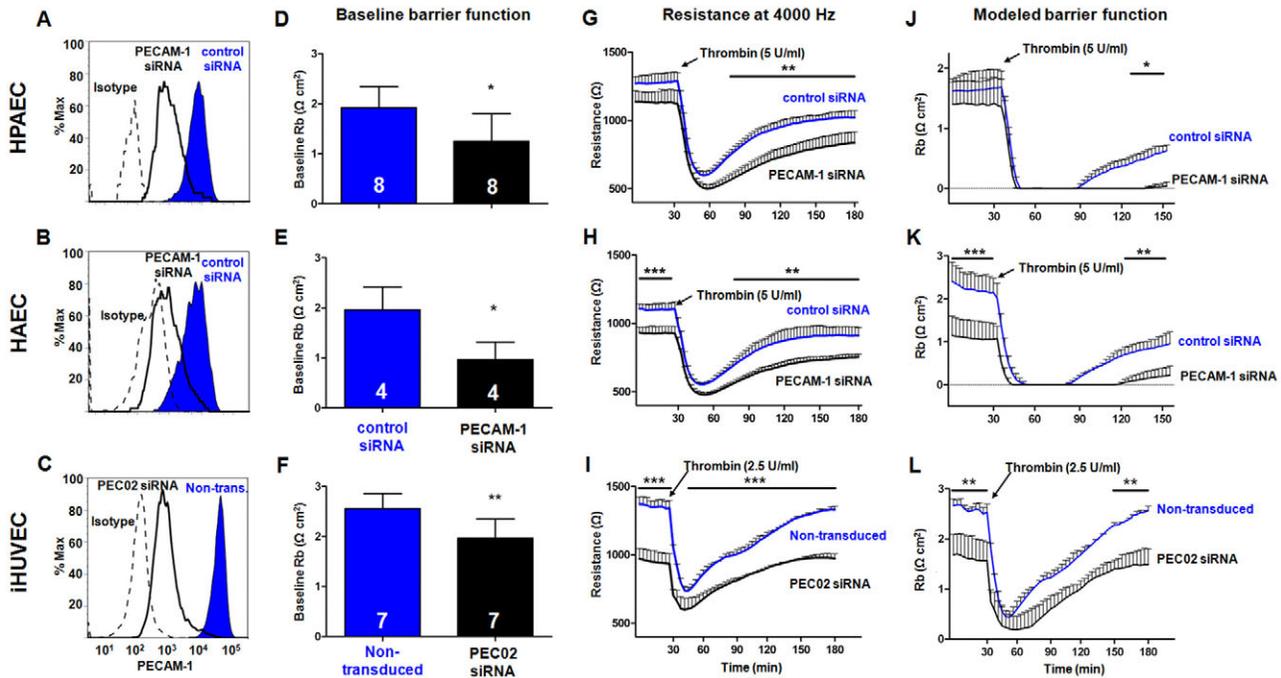


Fig. 1. Expression of PECAM-1 helps establish and restore vascular integrity. HPAECs (A) and HAECs (B) were transfected with control and *PECAM1* siRNA oligonucleotides. (C) iHUEVCs were either non-transduced or stably transduced with a lentivirus expressing PECAM-1-specific siRNA (PEC02). Expression of PECAM-1 was analyzed by flow cytometry and is indicated by lines in the histograms. The mean fluorescence intensity (MFI) of PECAM-1 expression within representative panels is as follows: (A) HPAECs: isotype, 65; *PECAM1* siRNA, 1762; control siRNA, 8288; (B) HAECs: isotype, 351; *PECAM1* siRNA, 1532; control siRNA, 7412; (C) iHUEVCs: isotype, 154; *PECAM1* siRNA, 1594; control siRNA, 41677. (D–F) Resistance to current flow at multiple frequencies was modeled by ECIS software to obtain the barrier function (R_b) parameter for endothelial monolayers at baseline before thrombin was added. Bars in graphs indicate the mean \pm s.d. of R_b in $\Omega \text{ cm}^2$ from the indicated number of wells for each group. Expression of PECAM-1 conferred significantly increased baseline barrier function in HPAECs (D), HAECs (E) and iHUEVCs (F) as determined by unpaired *t*-tests. HPAECs (G), HAECs (H), and iHUEVCs (I) were stimulated with thrombin and ECIS measurements were assessed at 4000 Hz. Lines in graphs report the mean \pm s.d. of resistance measurements in Ω versus time for the following: HPAECs and HAECs, three wells from one representative experiment of two independent experiments; iHUEVCs, two wells from one representative experiment of three independent experiments. Curves were determined to be significantly different from each other as assessed by repeated measures two-way ANOVA and are indicated in the panels. The R_b of HPAEC (J), HAEC (K) and iHUEVC (L) monolayers from representative experiments in G, H, and I, respectively, were obtained by modeling using ECIS software. Lines in graphs report the mean \pm s.d. of R_b in $\Omega \text{ cm}^2$ versus time. Curves were determined to be significantly different from each other as assessed by repeated measures two-way ANOVA and are indicated in the panels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

compared with PECAM-1_{Low} monolayers. These results were also seen in immortalized human umbilical vein endothelial cells (iHUEVCs), in which PECAM-1 had been stably silenced by lentivirally introduced siRNA (Fig. 1C,F). These results confirm and extend previous observations that PECAM-1 contributes to the maintenance of vascular barrier homeostasis in quiescent, non-challenged monolayers in which it is expressed.

To determine whether PECAM-1 also functions to restore vascular barrier integrity following perturbation of endothelial cell monolayers, we stimulated PECAM-1_{High} and PECAM-1_{Low} HPAECs, HAECs and iHUEVCs with thrombin and measured the rate of recovery of barrier integrity. As shown in Fig. 1G–I, PECAM-1_{High} monolayers displayed higher resistance to current flow at 4000 Hz – a value previously determined to be the optimal frequency at which to determine changes in barrier integrity (Giaever and Keese, 1991; Tiruppathi et al., 1992). Modeling of these thrombin-stimulated monolayers (Fig. 1J–L) revealed that recovery of barrier function following thrombin challenge was significantly delayed in PECAM-1_{Low} versus PECAM-1_{High} endothelial cells. These results demonstrate that, in addition to steady-state barrier maintenance, expression of PECAM-1 also

contributes importantly to the restoration of vascular barrier integrity after it has been compromised.

PECAM-1–PECAM-1 homophilic interactions are required to maintain steady-state endothelial cell barrier function

To identify the cellular mechanisms by which PECAM-1 maintains and restores vascular integrity, we created vectors expressing WT and variant forms of PECAM-1 that are deficient in its known adhesive and signaling properties (Fig. 2), expressed them in cells, and measured both their relative capacity to maintain steady-state barrier integrity, as well as their ability to restore barrier function after it had been perturbed. Two complementary approaches were used. In the first, we silenced endogenous PECAM-1 in primary HPAECs using the PECAM-1-specific siRNA PEC02 (Bergom et al., 2006), and then reconstituted expression with WT and variant forms of PECAM-1 containing silent mutations that rendered them resistant to silencing by PEC02 siRNA (Fig. 2) (Florey et al., 2010). As shown in Fig. 3A, HPAECs reconstituted with these variant forms of PECAM-1 expressed similar levels of PECAM-1. Most notably, cells expressing the K89A form of PECAM-1, which lacks homophilic-binding capacity (Newton et al., 1997),

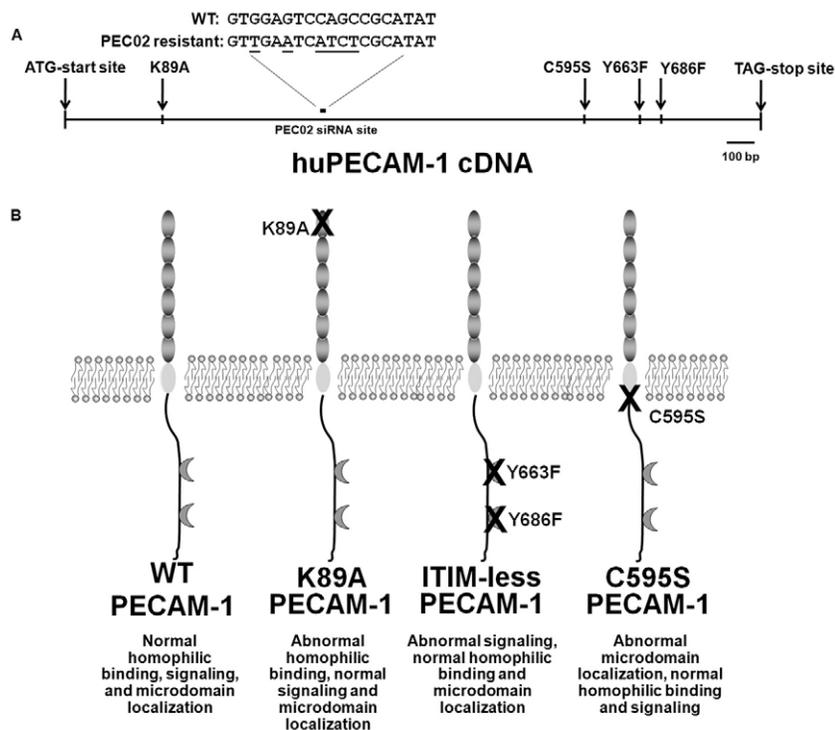


Fig. 2. Schematic of the wild-type and mutant forms of PECAM-1 used for barrier function assays. (A) Human *PECAM1* cDNA was mutated at specific amino acid residues to generate mutant forms of PECAM-1 as indicated. For experiments in which PECAM-1 expression was reconstituted with PEC02-resistant forms of PECAM-1, silent mutations were additionally made in the PEC02 siRNA binding site as indicated. (B) Schematic illustrating the deficiencies in adhesion, microdomain localization, or signaling of the mutant forms of PECAM-1.

displayed a markedly decreased baseline barrier function, which was similar to that of PECAM-1_{Low} HPAECs (pWPT control plasmid transfected). By contrast, HPAECs expressing signaling-deficient ITIM-less (Y663,686F) or raft-localization-deficient (C595S) PECAM-1 displayed normal baseline barrier functions that were comparable to that of HPAECs expressing WT PECAM-1 (Fig. 3B). Similar results were observed in REN mesothelioma cells expressing WT and variant forms of PECAM-1 (Fig. 3C,D). Taken together, these results demonstrate that homophilic adhesive interactions of PECAM-1, but not ITIM-mediated signaling or localization to membrane microdomains, have a significant role in both establishing and maintaining endothelial cell–cell junctional integrity.

Homophilic adhesive properties of PECAM-1 are required to re-establish vascular barrier integrity after thrombin stimulation

To determine whether the adhesive and/or signaling properties of PECAM-1 are required for restoration of vascular integrity, we stimulated HPAECs expressing the different forms of PECAM-1 with thrombin and measured their recovery using ECIS. As shown in Fig. 4A,B, cells expressing ITIM-less and C595S PECAM-1 responded to thrombin much like WT PECAM-1-reconstituted cells, whereas cells expressing K89A PECAM-1 displayed tracings that were similar to that of pWPT-transfected, PECAM-1_{Low} HPAECs. As an additional measure, we determined the slope of recovery in the tracings between the low point of barrier function and its return to baseline. As shown in Fig. 4C, K89A PECAM-1-expressing HPAECs were the only cells to exhibit a significantly decreased rate of recovery. These results demonstrate that, in addition to establishing and maintaining steady-state junctional stability, PECAM-1–PECAM-1 homophilic interactions are required for optimal restoration of vascular integrity following perturbation of endothelial cell–cell junctions.

Discussion

PECAM-1 is a well-studied adhesion and signaling receptor that has been shown to have reciprocal roles during the inflammatory response (Privratsky et al., 2010). PECAM-1 promotes inflammation by facilitating leukocyte transendothelial migration (Muller et al., 1993; Vaporciyan et al., 1993; Wakelin et al., 1996) and by serving as a mechanosensor for fluid shear stress (Tzima et al., 2005), but dampens inflammation via its ability to: (1) inhibit cellular activation (Falati et al., 2006; Newman et al., 2001; Newton-Nash and Newman, 1999; Patil et al., 2001; Rui et al., 2007; Wilkinson et al., 2002; Wong et al., 2002); (2) reduce pro-inflammatory cytokine levels (Carrithers et al., 2005; Goel et al., 2007; Maas et al., 2005; Tada et al., 2003); (3) decrease leukocyte accumulation at sites of inflammation (Carrithers et al., 2005; Goel et al., 2007; Goel et al., 2008; Maas et al., 2005; Tada et al., 2003); and (4) maintain and restore vascular integrity (Biswas et al., 2006; Carrithers et al., 2005; Ferrero et al., 1995; Graesser et al., 2002; Maas et al., 2005). Consequently, elucidating the mechanisms by which PECAM-1 mediates both its pro- and anti-inflammatory effects has the potential to improve our understanding of how cells and tissues integrate these seemingly opposing biological signals.

In the present investigation, we used ECIS technology to further characterize the properties of PECAM-1 that enable it to contribute to vascular barrier integrity. Two complementary approaches – siRNA-mediated silencing of PECAM-1-expressing endothelial cells and introduction of PECAM-1 into PECAM-1-negative endothelial-like cells – provided independent confirmation that PECAM-1_{Low} monolayers exhibit lower resistance to current flow than do PECAM-1_{High} monolayers, both at steady-state and following thrombin-induced vascular permeability (Figs 1, 3). These data demonstrate that PECAM-1 not only contributes to the maintenance of vascular integrity in resting, non-challenged cells, but also to its restoration following barrier disruption. These results correlate well with, and probably explain, previous observations

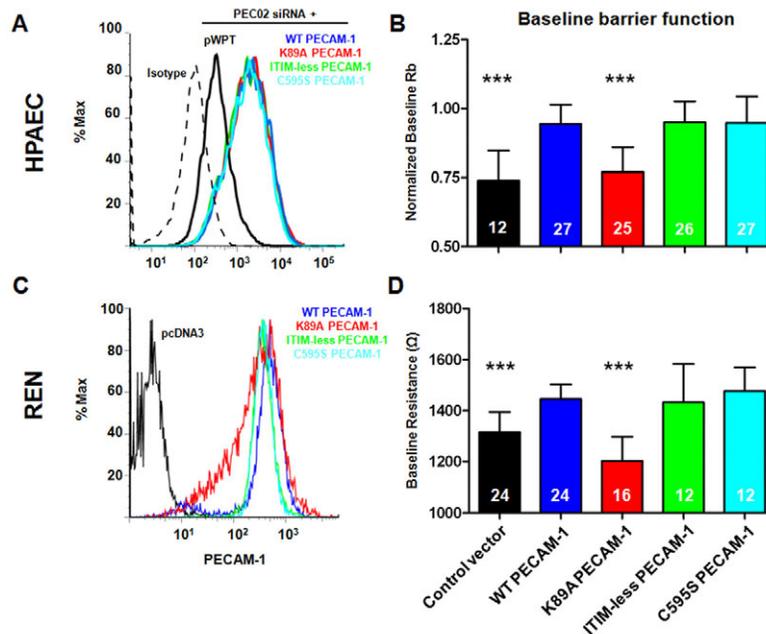


Fig. 3. Homophilic adhesive properties of PECAM-1 are required to establish barrier function at rest. (A) HPAECs were first transduced with lentivirus encoding PEC02 siRNA, sorted, and then transduced again with lentivirus encoding WT or mutant forms of PECAM-1 that were resistant to PEC02 siRNA or with pWPT (Control vector). Expression of PECAM-1 was assessed by flow cytometry and is indicated by the lines in the representative histograms. The PECAM-1 MFI for each cell type is as follows: Isotype, 118; pWPT, 483; WT, 2909; K89A, 2977; ITIM-less, 2706; C595S, 2527. (B) Baseline R_b of the various monolayers from A was obtained by modeling using ECIS software and then normalized to the WT-PECAM-1-transduced well with the highest baseline barrier function within its respective independent experiment. Bars in graph indicate the mean \pm s.d. of the normalized baseline R_b from the indicated number of wells analyzed for each group. HPAECs reconstituted with K89A PECAM-1 and pWPT displayed significantly lower baseline R_b compared with HPAECs transduced with WT PECAM-1 as determined by one-way ANOVA. (C) REN mesothelioma cells were transfected with pcDNA3 control plasmid (Control vector), or plasmids encoding WT or mutant forms of PECAM-1, and expression of PECAM-1 was assessed by flow cytometry. The MFI of PECAM-1 expression is as follows: pcDNA3, 3; WT, 497; K89A, 419; ITIM-less, 400; C595S, 398. (D) Baseline resistance of the monolayers from C to current flow at 4000 Hz was assessed by ECIS, and bars in the graphs report the mean \pm s.d. of the baseline resistance in Ω from the indicated number of wells for each group. Cells expressing K89A PECAM-1 again had significantly lower baseline resistance to current flow as determined by one-way ANOVA. *** $P < 0.001$ vs WT PECAM-1.

that PECAM-1-deficient mice exhibit compromised vascular integrity following (1) vascular injury (Mahooti et al., 2000), (2) LPS-induced endotoxic challenge (Carrithers et al., 2005; Maas et al., 2005), (3) induction of EAE in the brain (Graesser et al., 2002), and (4) histamine challenge in the skin (Graesser et al., 2002).

To establish the functional properties of PECAM-1 necessary for its barrier function, we introduced mutant forms of PECAM-1 lacking key function-determining residues into PECAM-1-deficient endothelial cell lines and examined their contribution to barrier integrity, both at rest and following disruption, using ECIS technology. Interestingly, HPAECs expressing a homophilic binding-crippled K89A mutant form of PECAM-1 exhibited poor barrier function under both conditions, whereas cells expressing ITIM-less and C595S forms of PECAM-1 were able to maintain and restore barrier integrity similarly to that of HPAECs expressing wild-type PECAM-1 (Figs 3, 4). The inability of K89A PECAM-1 to become concentrated at endothelial cell–cell junctions (supplementary material Fig. S1) (Bergom et al., 2008; Sun et al., 2000), probably contributes to the inefficiency with which it is able to maintain or restore vascular barrier integrity, and confirms the importance of PECAM-1–PECAM-1 homophilic interactions in controlling both the subcellular location and vascular function of this adhesion and signaling receptor. Because engagement of PECAM-1 results in activation of integrin on leukocytes (Berman et al., 1996; Berman and Muller, 1995; Dangerfield et al., 2002; Tanaka et al., 1992; Vernon-Wilson et al., 2006; Vernon-Wilson et

al., 2007), platelets (Varon et al., 1998) and endothelial cells (Chiba et al., 1999), and because integrins enable endothelial cells to bind to extracellular matrix proteins at focal adhesions, which are crucial determinants of cell shape and permeability (Mehta and Malik, 2006), it will be interesting in future studies to determine whether integrin activation downstream of homophilic PECAM-1 engagement has a role in PECAM-1-mediated maintenance of vascular barrier integrity. If it does, then PECAM activation of integrins in this case does not involve the traditional signaling through its ITIM domains (Figs 3, 4).

The PECAM-1 cytoplasmic domain has previously been implicated in the regulation of endothelial cell junctional permeability via its ability to form a ternary complex with the protein tyrosine phosphatase SHP-2 (Biswas et al., 2006) and tyrosine phosphorylated β -catenin (Biswas et al., 2005). In this proposed mechanism, the SH2 domains of SHP-2 associate with phosphorylated ITIM tyrosines in PECAM-1, encoded by exons 13 and 14, whereas β -catenin binds PECAM-1 at a nearby downstream site encoded by exon 15, facilitating dephosphorylation of β -catenin. Dephosphorylated β -catenin would then be free to rebind VE-cadherin, reconstitute the adherens junction and stabilize the permeability barrier. Despite the attractiveness of this proposed mechanism, our finding (Figs 3, 4) that ITIM-less PECAM-1 was as effective as wild-type PECAM-1 in both maintaining and restoring endothelial cell junction integrity following exposure to thrombin makes it likely that ITIM-independent mechanisms are

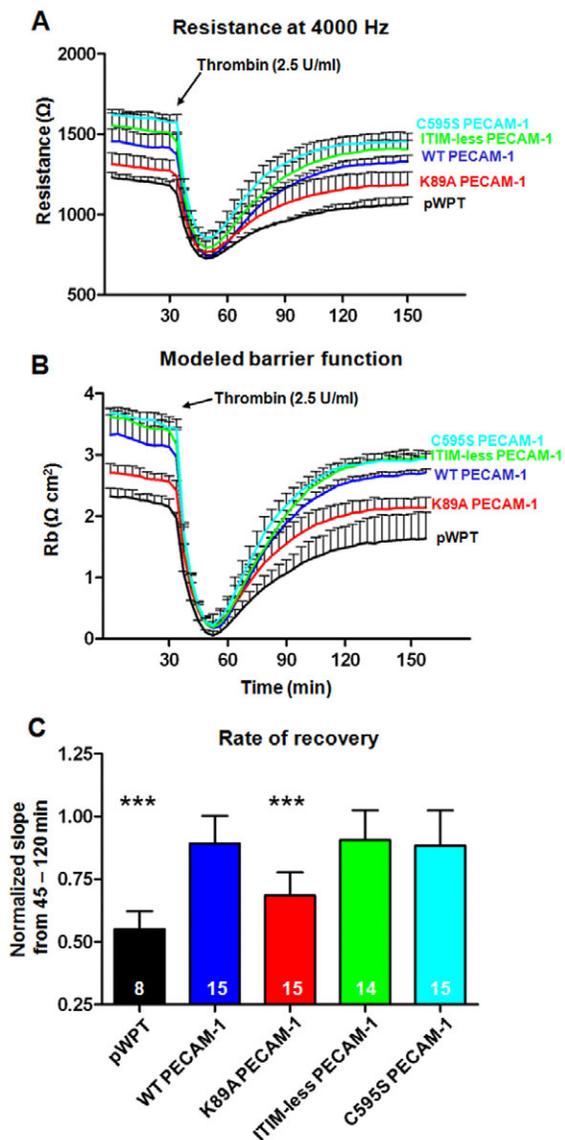


Fig. 4. Homophilic adhesive properties of PECAM-1 are required to restore vascular barrier integrity after thrombin challenge. (A) HPAECs were first transduced with lentivirus containing PEC02 siRNA, sorted, and then transduced again with lentivirus encoding WT and mutant forms of PECAM-1 that were resistant to PEC02 siRNA (representative expression is shown in Fig. 3A). ECIS measurements at 4000 Hz were obtained on cells stimulated with thrombin, and lines in the graph display the mean \pm s.d. of the resistance in Ω versus time for three wells in one representative experiment. Similar results were obtained in a four other independent experiments. (B) ECIS measurements were modeled to obtain the R_b of monolayers, and lines in the graph displays the mean \pm s.d. of the R_b in Ωcm^2 versus time for three wells in the representative experiment in A. (C) The slope of R_b curves from the lowest point to a point near full recovery was obtained by linear regression to assess the rate of recovery. Each well that was analyzed was normalized to the well expressing WT PECAM-1 with the highest slope within its respective independent experiment (five independent experiments). Results from the indicated number of wells are reported as the mean \pm s.d. of the normalized slope of recovery between the indicated time periods. HPAECs that were reconstituted with K89A PECAM-1 had a significantly decreased slope of recovery compared with the other cell types as assessed by one-way ANOVA. *** P <0.001 vs WT PECAM-1.

also involved. Further studies will be required to characterize the ITIM-independent regions of the PECAM-1 cytoplasmic domain that are involved in PECAM-1-mediated barrier-promoting activities.

S1P is a bioactive lipid that stabilizes endothelial cell junctions via its binding to the G-protein-coupled receptor S1P1 (Komarova et al., 2007; Rosen and Goetzl, 2005). A number of potentially interesting links have been reported to exist between PECAM-1 and S1P biology. First, PECAM-1 has been reported to both bind to (Fukuda et al., 2004) and modulate the function of (Limaye et al., 2005) sphingosine-1-kinase, a lipid-raft-localized enzyme that phosphorylates sphingosine to yield S1P (Rosen and Goetzl, 2005). S1P, in turn, appears to be able to induce phosphorylation of PECAM-1 ITIMs (Huang et al., 2008). Finally, the fraction of PECAM-1 that is localized to lipid rafts has been found to influence the ability of S1P1 to signal (Gratzinger et al., 2003). Taken together, these data suggest that the fraction of PECAM-1 that exists in lipid rafts might function to stabilize endothelial cell-cell junctions via its ability to localize sphingosine-1-kinase to rafts, where the S1P generated can bind to proximal raft-localized S1P1, thereby increasing barrier function. Our finding (Figs 3, 4) that the C595S PECAM-1 mutant form of PECAM-1 is as efficient at stabilizing endothelial cell-cell junctions as is wild-type PECAM-1, however, suggests that PECAM-1-mediated coordination of S1P signaling, if it occurs, does not require its raft localization. Owing to the importance of S1P in vascular barrier function and the ability of S1P and PECAM-1 to regulate each other, greater clarity is needed as to how and/or whether PECAM-1 promotes barrier maintenance via its effects on S1P signaling.

In conclusion, we have demonstrated that homophilic PECAM-1-PECAM-1 interactions and its localization to cell-cell junctions are primarily responsible for PECAM-1-mediated barrier protection. By contrast, PECAM-1-mediated signaling through cytoplasmic ITIMs, and its localization to membrane microdomains, appear to be dispensable for its barrier protective properties. Expression of PECAM-1 has been shown to influence disease outcomes in model systems that demonstrate changes in permeability, either acutely as in endotoxic shock (Carrithers et al., 2005; Maas et al., 2005), or chronically as in atherosclerosis (Goel et al., 2008; Harry et al., 2008). The barrier protective properties of PECAM-1 have also been implicated in the accumulation of leukocytes at inflammatory sites (Graesser et al., 2002), which influences tissue and organ damage during inflammatory responses. Consequently, elucidation of the mechanisms by which PECAM-1 regulates barrier function will have important implications for understanding the pathological processes in both acute and chronic inflammatory diseases.

Materials and Methods

Cell lines

All cell culture reagents were obtained from Mediatech (Manassas, VA), unless otherwise specified. HPAECs and HAECs were obtained from Invitrogen (Carlsbad, CA) and maintained in Medium 200 (Invitrogen) supplemented with low serum growth supplement (Invitrogen) and 400 $\mu\text{g}/\text{ml}$ gentamycin. iHUVECs were generated by transducing HUVECs with the recombinant retrovirus LXSN16 E6/E7 as previously described (Moses et al., 1999). iHUVECs were maintained in human endothelial serum-free medium (Invitrogen), 10% FBS (Sigma, St Louis, MO), 5% human AB serum (Invitrogen), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 150 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement (BD Biosciences, San Jose, CA). REN mesothelioma cells (Smythe et al., 1994) were maintained in RPMI 1640, 10% FBS, 2 mM L-glutamine, and 500 $\mu\text{g}/\text{ml}$ gentamycin. Stable REN cell lines expressing WT and K89A PECAM-1 have been previously described (Sun et al., 2000). REN cells expressing ITIM-less (Y663,686F) and C595S PECAM-1 were generated by transfecting REN cells (Lipofectamine 2000, Invitrogen) with a pcDNA3 plasmid encoding human PECAM-1 with the tyrosines at 663 and 686 mutated to phenylalanine, and the cysteine residue at 595 mutated to serine, respectively, using

a site-directed mutagenesis kit (Stratagene, La Jolla, CA). All stable REN cell lines were supplemented with G418 (0.6 mg/ml) to maintain stable expression of PECAM-1.

ECIS measurements

ECIS measurements (Giaever and Keese, 1991; Tiruppathi et al., 1992) were performed in 8W10E+ electrode arrays on an ECIS Z θ instrument (Applied Biophysics, Troy, NY). Cells were plated on arrays coated with Attachment Factor (Invitrogen) and allowed to grow to confluence for 3–4 days. On the day of the experiment, culture medium was replaced with 360 μ l of medium as follows: REN cells, REN medium diluted 1:10 with RPMI 1640; iHUEVCs, iHUEVC medium diluted 1:10 with human endothelial SFM; HPAECs and HAECs, non-diluted medium from above. Cells were allowed to achieve a stable baseline and then stimulated with 40 μ l of respective medium containing 10 \times concentration of human thrombin (Sigma, T4393) (1 \times final concentration of thrombin as shown in figures). ECIS measurements were recorded at multiple frequencies and modeled with ECIS software (Applied Biophysics) to obtain the barrier function parameter *R_b*, which is expressed as the average basal electrical resistance (in units of Ω cm²). Briefly, through the use of established formulas, and the assumption that cells can be represented as disk-shaped objects that have insulating membrane surfaces and that are filled with conducting electrolyte, ECIS software is able to model three separate parameters – membrane capacitance, current flow under cells, and current flow between cells – when the resistance and capacitance of confluent monolayers are measured at multiple frequencies (Giaever and Keese, 1991). This ECIS model allows a sensitive determination of the amount of current that is actually passing between cells, the true resistance of the barrier (*R_b*), which is a robust reporter of barrier function. In REN cell experiments, resistance (in units of Ω) at 4000 Hz is reported. Graphs that depict the baseline barrier function report the averaged *R_b* within each cell group at the time thrombin was added to wells. In some experiments, baseline barrier function was normalized within experiments to combine results from multiple experiments. For normalization, baseline barrier function of each well was normalized to the WT PECAM-1-transduced well with the highest baseline barrier function within each independent experiment. Graphs depicting the rate of recovery report the average of the slope of the barrier function curves from individual wells for the indicated time periods that were obtained by linear regression and then normalized to the WT PECAM-1-expressing well with the highest slope within each independent experiment.

Transfection of siRNA

HPAECs and HAECs were transfected with control siRNA-A (25 nM) or *PECAM1* siRNA (25 nM) using Santa Cruz transfection reagent and transfection medium (Santa Cruz Biotechnologies, Santa Cruz, CA) according to the manufacturer's instructions. All transfections were performed in individual wells of ECIS arrays. Both control siRNA-A and *PECAM1* siRNA consist of pools of three siRNA oligonucleotides obtained from Santa Cruz Biotechnologies. The level of *PECAM1* silencing was determined by dividing the residual amount of remaining PECAM-1 in siRNA-transduced cells by the amount of PECAM-1 in control-siRNA-transduced cells. This percentage was then subtracted from 100% to obtain the amount of *PECAM1* silencing. *PECAM1* siRNA silenced expression of endogenous PECAM-1 by 65–80% in HPAECs and 65–85% in HAECs compared with control siRNA-A-treated cells over multiple experiments (represented in Fig. 1A,B). ECIS measurements were obtained on cells 3 days after transfection.

Generation of lentiviral constructs encoding PEC02-resistant forms of PECAM-1

Full-length human *PECAM1* was inserted into the lentiviral plasmid pWPT–GFP (Addgene #12255) in place of eGFP and was used as a template to make PECAM-1 rescue constructs as previously described (Florey et al., 2010). In brief, six silent mutations within the PEC02 siRNA target region were introduced using site-directed mutagenesis (Stratagene) with the following oligonucleotide primers: (1) Fwd, 5'-CTACACGTGCAAAGTTGAATCATCTCGCATATCC-3'; (2) Rev, 5'-GGATA-TGCGAGATGATTCAACTTTGCACGTGTAG-3' (silent mutation sites are highlighted). Further mutagenesis was carried out by introducing a lysine to arginine mutation at position 89, tyrosine to phenylalanine mutations at positions 663 and 686, or a cysteine to serine mutation at position 595. All lentiviruses were generated by the Viral Vector Core facility at the Blood Research Institute.

Lentiviral transduction of iHUEVCs and HPAECs

iHUEVCs and HPAECs were transduced with lentiviruses expressing the *PECAM1*-specific siRNA PEC02 (Bergom et al., 2006). Because these lentiviruses also express a selectable eGFP marker, which is driven off of a separate promoter, both cell types were sorted for eGFP expression using a BD FACS ARIA cell sorter (BD Biosciences) to obtain a homogenous PECAM-1_{LOW} population. PEC02 siRNA silenced expression of endogenous PECAM-1 by 96% in iHUEVCs (Fig. 1C) and by 80–90% in HPAECs (Fig. 3A, histogram of non-transduced cells not shown) as described above. HPAECs that stably expressed PEC02 siRNA were further plated in ECIS arrays, or gelatin-coated and glutaraldehyde-crosslinked (Sigma) eight-chamber glass slides (BD Biosciences) and re-transduced with lentiviruses expressing pWPT control virus, non-PEC02-resistant PECAM-1 or PEC02-resistant WT and mutant forms of

PECAM1 to reconstitute *PECAM1* expression. PECAM-1-reconstituted cells were used in ECIS or confocal experiments at 3–4 days after transduction. HPAECs were used up to passage 9 and iHUEVCs were used between passages 21 and 28.

Flow cytometry for PECAM-1 expression

Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences). For REN and iHUEVCs, cells were lifted with trypsin-EDTA and stained with Alexa-Fluor-647–PECAM-1.3, which was created by labeling mAb PECAM-1.3 with the Alexa Fluor 647 monoclonal antibody labeling kit (Molecular Probes, Carlsbad, CA) according to the manufacturer's instructions. For HPAECs and HAECs, where transfections and transductions were performed in ECIS arrays, cells were lifted with trypsin-EDTA after ECIS measurements were recorded, stained with Alexa-Fluor-647–PECAM-1.3, and analyzed by flow cytometry.

Confocal imaging

Confluent monolayers of HPAECs from above were fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with PBS containing 0.3% BSA. Monolayers were then incubated sequentially with anti- β -catenin (1:200, Cell Signaling #2677), Alexa Fluor 405 anti-mouse IgG (1:200, Invitrogen), biotin-conjugated PECAM-1.3 at 5 μ g/ml, streptavidin-conjugated Alexa Fluor 647 (1:200, Invitrogen) with three washes between each antibody incubation. Slides were mounted with ProLong Gold antifade reagent (Invitrogen), and images were obtained using a 40 \times oil objective and lasers at 405 and 635 nm on a FluoView FV1000 multi-photon emission microscope (Olympus, Center Valley, PA). Z-stack fluorescent images were acquired at 8 μ s per pixel at optimal stepwise progression through the whole thickness of the monolayer. Images were deconvoluted (nearest neighbors) and analyzed with SlideBook 5.0 imaging software (Leeds Precision Instruments, Minneapolis, MN). Since residual endogenous PECAM-1 was still present at cell–cell borders in all cell types after silencing of *PECAM1*, we chose to quantify the amount of non-border-localized PECAM-1 to determine the relative localization of the PECAM-1 mutants. To do this, a universal mask, based on the β -catenin channel (expressed at cell–cell borders), was generated to maximize exclusion of all cell–cell border voxels, and was applied to all images. The mean voxel intensity of non-border localized PECAM-1 was normalized to mean voxel intensity of total PECAM-1 through the whole monolayer for each image. This result was further normalized within independent experiments to the WT PECAM-1-expressing image with the highest non-border PECAM-1 intensity. For representative images, a projection image of combined z-stacks was created using SlideBook 5.0 software, and brightness and contrast were adjusted uniformly for all images in Microsoft PowerPoint.

Statistical analysis

Results are expressed as mean \pm s.d. Statistical analysis was performed on GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/124/9/1477/DC1>

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