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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 (ΔCD-PECAM-1). The endothelial barrier function was evaluated by Electric Cell-substrate Impedance Sensing, and molecular mobility was assessed by fluorescence recovery after photobleaching.

Key findings: We found that ΔCD-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 ΔCD-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.

1. 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 [2,37,39]. 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 [43,51,52]. Extracellular domain-mediated homophilic binding is critical for concentrating PECAM-1 at endothelial cell-cell junctions [50,55], 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 [9,17,21,32,33] and in vitro [30,35,45].

The PECAM-1 cytoplasmic domain is encoded by eight exons [27], is largely unstructured [42], and carries out multiple functions in endothelial cells. Specifically, it is required for PECAM-1 to (1) function as part of a mechanosensory complex [11,13,26,54] (2) confer cytoprotection in response to proapoptotic stimuli [4,16,19], and (3) interact with other junctional adhesion proteins and cytoskeletal molecules [5–7,22,23,54]. 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 [50,55]. Little is known,

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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.

2. Results

2.1. 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 [1,15], monkey Cos7 cells [1], Chinese hamster ovary cells [55], murine L-cells [1,51], murine brain endothelioma cells [54,55], bovine aortic endothelial cells [54], and human mesothelioma cells [50]. 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 iHUVECs), and one in which only the PECAM-1 cytoplasmic domain has been deleted (ΔCD-PECAM-1 iHUVECs). 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 iHUVECs were produced by transducing iHUVECs 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. ΔCD-PECAM-1 iHUVECs 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 [47], 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.

2.2. 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 iHUVECs lacked PECAM-1 expression, while the ΔCD-PECAM-1 iHUVECs expressed the extracellular, but not cytoplasmic, domain of PECAM-1. As expected, wild-type iHUVECs bound both mAbs (Fig. 2A), ΔCD-PECAM-1 bound only mAb PECAM-1.3 (Fig. 2B), while KO-PECAM-1 iHUVECs bound neither (Fig. 2C). Confocal microscopy was then employed to assess the ability of wild-type PECAM-1

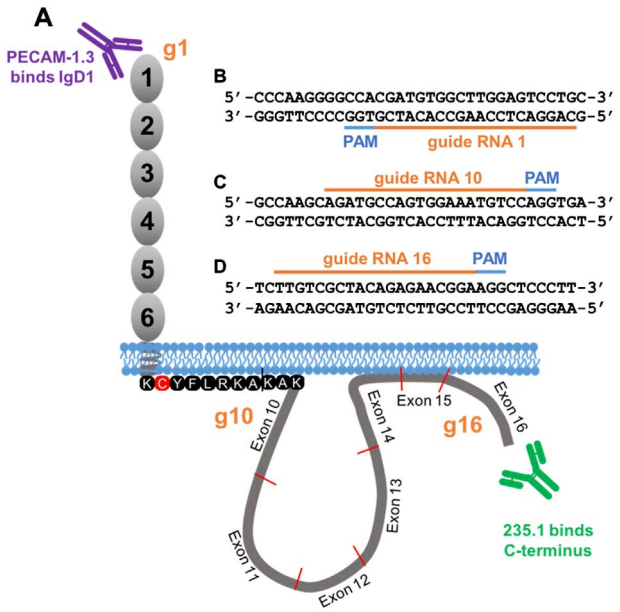

Fig. 1. Strategy used to generate PECAM-1 knockout and cytoplasmic domain-deleted iHUVEC cell lines. (A) Schematic of PECAM-1 showing the locations of antibody binding sites for mAb PECAM-1.3, specific for PECAM-1 IgD1, and mAb 235.1, specific for the C-terminus of the PECAM-1 cytoplasmic domain. (B) 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 (ΔCD-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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2D–F) and ΔCD-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 ΔCD-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.

2.3. 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 [30,35,45], 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 [20], was used to determine whether the PECAM-1 cytoplasmic domain plays a role in regulating vascular permeability. iHUVEC cell lines expressing WT-, ΔCD-, 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 iHUVECs 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, ΔCD-PECAM-1 iHUVECs 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.