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Role of lateral cell–cell border location and extracellular/transmembrane domains in PECAM/CD31 mechanosensation

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Keywords
Platelet endothelial adhesion molecule-1, Endothelial mechanotransduction, Hyperosmotic stress, Fluid shear stress

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Role of lateral cell–cell border location and extracellular/transmembrane domains in PECAM/CD31 mechanosensation ☆

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Abstract

Phosphorylation of tyrosine residues on platelet–endothelial cell adhesion molecule-1 (PECAM-1), followed by signal transduction events, has been described in endothelial cells following exposure to hyperosmotic and fluid shear stress. However, it is unclear whether PECAM-1 functions as a primary mechanosensor in this process. Utilizing a PECAM-1–null EC-like cell line, we examined the importance of cellular localization and the extracellular and transmembrane domains in PECAM-1 phosphorylation responses to mechanical stress. Tyrosine phosphorylation of PECAM-1 was stimulated in response to mechanical stress in null cells transfected either with full length PECAM-1 or with PECAM-1 mutants that do not localize to the lateral cell–cell adhesion site and that do not support homophilic binding between PECAM-1 molecules. Furthermore, null cells transfected with a construct that contains the intact cytoplasmic domain of PECAM-1 fused to the extracellular and transmembrane domains of the interleukin-2 receptor also underwent mechanical stress-induced PECAM-1 tyrosine phosphorylation. These findings suggest that mechanosensitive PECAM-1 may lie downstream of a primary mechanosensor that activates a tyrosine kinase.

Keywords: Platelet endothelial adhesion molecule-1; Endothelial mechanotransduction; Hyperosmotic stress; Fluid shear stress

Mechanical stresses, including fluid shear stress (FSS), play an important role in determining endothelial cell (EC) behavior, modulating their physiology, gene expression, and morphology [1,2]. Transfer of FSS forces to the EC first occurs at the luminal cell surface where molecules whose conformations are directly affected by FSS may act as mechanosensors or mechanotransducers. In addition, sites remote from the initial stimulus may act as mechanosensors or mechanotransducers as the force of FSS is transmitted throughout the cell via the cytoskeleton [1]. One such location is the lateral cell–cell adhesion site [3].

Recently, investigators have identified a possible role for platelet–endothelial cell adhesion molecule-1 (PECAM-1, CD31) in the sensation of hyperosmotic stress (HOS) and FSS and subsequent signal transduction events [4–6]. PECAM-1 is a 130-kDa member of the immunoglobulin superfamily that is expressed abundantly on the cell surface of ECs, platelets, and many leukocytes. A striking feature of PECAM-1 is its localization at the cell–cell border between adjacent endothelial cells [7,8]. This specific localization may be important to the vascular function of PECAM-1, playing a role in leukocyte transmigration of EC monolayers [9]. In confluent endothelial cells, PECAM-1 molecules on adjacent cells bind homophilically to each other via extracellular domains 1 and 2 [10].

☆ *Abbreviation:* PECAM-1, platelet–endothelial cell adhesion molecule-1.

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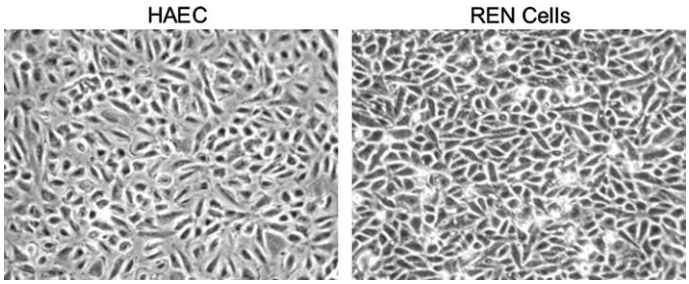

Fig. 1. Morphology of HAEC (left) and REN cells (right) in culture. Cells were cultured on gelatin-covered glass microscope slides and grown to confluence 48 h after seeding. Photomicrographs were obtained just prior to application of shear stress in a parallel plate flow chamber (described in [18]).

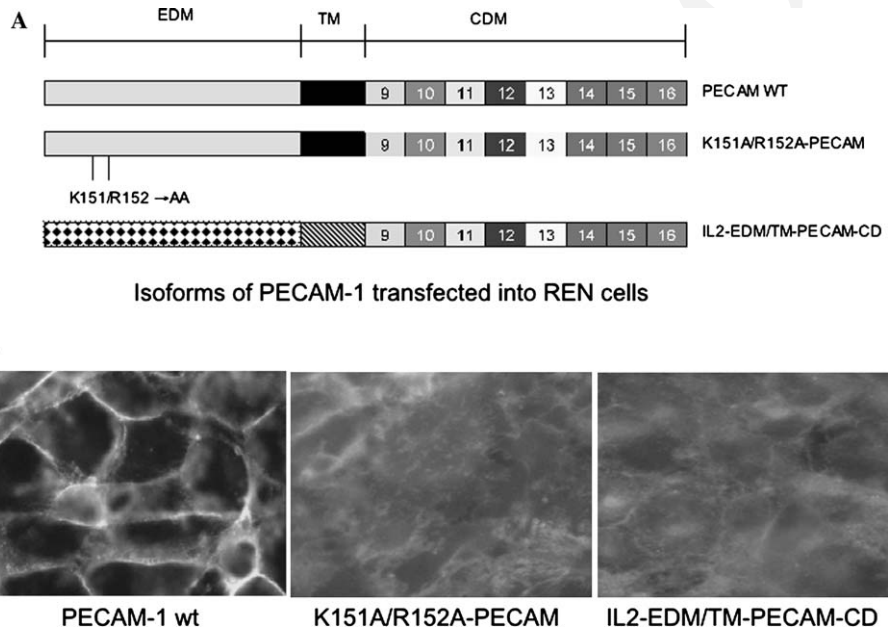

Fig. 2. (A) Isoforms of PECAM-1 transfected into REN cells. (B) Distribution of PECAM-1 isoforms transfected into REN cells. (Left panel) Wild-type PECAM-1 localizes to the lateral cell–cell border. (Center panel) The K151A/R152A-PECAM mutant is found diffusely on the cell membrane, as is the IL2-EDM/TM-PECAM-CD mutant (right panel).

REN cells are endothelial-like cells derived from human malignant mesothelioma [11]. In culture, they form a confluent monolayer and adopt a "cobblestone" morphology reminiscent of ECs (Fig. 1). In addition, REN cells express several surface antigens in common with ECs but lack PECAM-1; they can be transfected stably with wild-type or mutant forms of PECAM-1 (Fig. 2A). We have previously used REN cells as an EC model, finding that many EC signaling processes may be reconstituted after PECAM-1 expression [12,13].

Utilizing this null cell, we reasoned that if PECAM-1 is a mechanosensor, force-induced PECAM-1 phosphorylation may require localization to, and organization at, the lateral cell–cell border. We also explored whether the cytoplasmic, extracellular or transmembrane domains of PECAM-1 are necessary for PECAM-1 mechanosignaling.

Materials and methods

Antibodies, reagents, immunoprecipitation, and Western blotting. Antibodies included the following: mAb 4G6, a murine immunoglobulin (IgG) directed against the PECAM-1 extracellular Ig loop six domain [14]; mAb 1.3, a murine IgG directed against the PECAM-1 extracellular domain (a gift of Dr. Peter Newman, Blood Center of Southeastern Wisconsin, Milwaukee, WI); PCD, a rabbit polyclonal antibody directed against the PECAM-1 cytoplasmic domain; ab8325 (Abcam, Cambridge, UK), a murine mAb directed against the α-subunit of the interleukin-2 receptor (IL2R); anti-SHP-2 mAb (Cell Signaling Technology, Santa Cruz, CA); and PY20 (Transduction Laboratories, BD Biosciences, Palo Alto, CA), an anti-phosphotyrosine rabbit polyclonal Ab. Purified antibodies were obtained by protein G affinity chromatography of hybridoma supernatants or serum. Active binding of antibodies was confirmed by flow cytometry.

For immunoprecipitation, thawed lysates were preabsorbed with protein A-conjugated Sepharose beads (Amersham–Pharmacia). After removal from the beads, the precleared supernatants were transferred to fresh microfuge tubes and immunoprecipitated by incubation with mAb 4G6 (for WT PECAM-1 and the K151/R152A mutant) or

ab8325 (for the IL2PCD construct), followed by incubation with protein A-conjugated Sepharose beads.

Lysates were then separated on 4–12% gradient SDS–polyacrylamide reducing gels (Invitrogen) and transferred to PVDF membranes (Millipore). Membranes were probed with mAb 1.3 or pAb PCD and then counterstained with HRP-conjugated donkey anti-mouse IgG (Cappel) or HRP-conjugated goat-anti-rabbit IgG (Jackson), and signals were visualized with ECL (Amersham–Pharmacia). Membranes were then stripped in a buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, and 100 mM of 2-mercaptoethanol, then reprobed with PY20 and counterstained with HRP-conjugated goat-anti-rabbit IgG. Signals were again detected by ECL. Images were captured on a desktop scanner (Canon CanoScan D1250U2F) utilizing Adobe Photoshop 7.0.

Cell lines and mutant PECAM-1 constructs. Human aortic endothelial cells (HAEC, Clonetics) were cultured in endothelial basic medium-2 (EBM-2, Clonetics) containing 2% fetal bovine serum and Bullet kit reagents (Clonetics). Only HAECs between passages 2 and 6 were used.

REN cells, a human mesothelioma cell line previously isolated in our laboratories [11], were grown in RPMI (Gibco) supplemented with 10% FBS and 2 mM L-glutamine (R10 media) containing 10,000 U penicillin and 10,000 U streptomycin. PECAM-1 mutant constructs IL2PCD and K151/R152A [15] as well as wild-type PECAM-1 were subcloned into the pcDNA-neo vector and transfected into REN cells. Expression was subsequently confirmed by flow cytometry (Coulter) as described previously [12]. Stable polyclonal populations of REN cell transfectants were generated by bead sorting (Dynal) and selected in G418 (0.5 mg/mL) supplemented R10 media as previously described [13].

The IL2PCD PECAM-1 mutant contains the extracellular and cytoplasmic domains of the interleukin-2 receptor fused to the full cytoplasmic domain of PECAM-1 [15]. The K151/R152A mutant contains mutations lysine–arginine (KR) at amino acid positions 151 and 152 to alanine–alanine in the putative glycosaminoglycan binding region of PECAM-1 (amino acids 149–155, see [16]). Previously, these mutant forms of PECAM-1 have been demonstrated to spread diffusely over the cell surface rather than localize to lateral cell–cell adhesion junctions, and we confirmed these observations in the cell lines used for these experiments (Fig. 2B) [15].

Immunofluorescent staining. Cells were grown on gelatin-coated coverslips, washed in phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde for 20 min, and then permeabilized with ice-cold 0.5% NP-40 for 1 min. After washing, cells were stained using anti-PECAM-1 mAb 4G6 and polyclonal antibody "PCD" (directed against the cytoplasmic domain of PECAM-1) as previously described [17]. Cells were viewed on a Nikon eclipse E400 fluorescence microscope using a 40× oil fluorescence lens and photographed with a Nikon Coolpix 4500 digital camera.

Hyperosmotic stress and fluid shear stress. Cells were seeded onto gelatin-coated glass microscope slides 48 h prior to the experiment and grown to confluence. For experiments with HAECs, EBM-2 (Clonetics) containing 2% fetal bovine serum and Bullet kit reagents (Clonetics), supplemented with 1% dextran, was used. To enhance the PY-PECAM-1 signal, this medium was supplemented with 5 mM $NaVO_3$ (pH 7.4); HAECs incubated for 3 h in $NaVO_3$-containing medium were used as a positive PY-PECAM-1 control.

REN cells were incubated in R10 medium containing 1% dextran and 5 mM $NaVO_3$ (pH 7.4) at 37 °C for 2 h prior to exposure to mechanical stress. For FSS, glass slides were placed in a parallel plate flow chamber [18] and subjected to 13 dyn/cm^2 of continuous shear stress for 15 min with cell growth media supplemented with 1% dextran (to increase the media's viscosity) and 5 mM $NaVO_3$. For HOS, cells were exposed to medium containing 1% dextran, 5 mM $NaVO_3$, and 600 mM sucrose. After mechanical stress, cells were washed twice with ice-cold PBS containing 1 mM $NaVO_3$ and lysed for 20 min on ice with a buffer containing 0.01 M Tris–acetate (pH 8.0), 0.5% NP-40, 0.5 mM Ca^{2+}, 10 mcg/mL leupeptin, 10 mcg/mL aprotinin, 2 mM PMSF, and 2 mM $NaVO_3$. Lysates were centrifuged at 14,000*g* for 10 min at 4 °C and the supernatant was stored at −80 °C.

Results and discussion

Fluid shear stress leads to tyrosine phosphorylation of PECAM-1 in HAECs and REN cells transfected with wild-type PECAM-1

FSS and HOS induce tyrosine phosphorylation of PECAM-1 in cultured bovine aortic endothelial cells [5,6]. To confirm this observation, and to ascertain whether this phenomenon is present in human EC, physiologic FSS (13 dyn/cm^2) was applied for 15 min, or HOS for 10 min, to cultured HAECs. PECAM-1 purified from HAECs subjected to FSS demonstrated significantly higher levels of tyr-P than controls (Fig. 3). We also confirmed the observation that PECAM-1–tyr-P co-immunoprecipitated with SHP-2, as observed by other investigators (data not shown) [19].

In order to explore the role of PECAM-1 as a mechanosensitive molecule in depth, we chose the REN cell model as a null cell. Because wild-type-PECAM-1 is expressed abundantly on all known lines of ECs, detecting the effects of mutations to PECAM-1 is difficult. Some investigators have employed anti-sense s-oligo techniques to knock down the expression of wt-PECAM-1, but such techniques only suppress the expression of wt-PECAM-1 to approximately 70% of normal [5]. Thus, we subjected REN cells transfected with wt-PECAM-1 (REN-HP) to FSS and HOS. In order to strengthen the PECAM-1–tyr-P signal in Western blotting, phosphatase activity was inhibited by incubating the cells with growth media containing 5 mM $NaVO_3$ for 2 h prior to FSS or HOS (control samples were incubated with growth media containing 5 mM $NaVO_3$ for 2 h 15 min). FSS or HOS stimulated tyr-P of PECAM-1

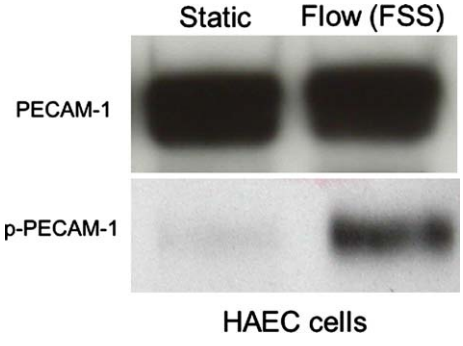

Fig. 3. Three hour incubation with 5 mM sodium vanadate and 15 min of fluid shear stress (FSS) lead to tyrosine phosphorylation of PECAM-1 in HAECs. Cell lysates were immunoprecipitated with anti-PECAM-1 mAb 4G6 and membranes were blotted with anti-PECAM-1 mAb 1.3 (upper panel). Membranes were then stripped and reprobed with anti-PY pAb PY20 (lower panel).

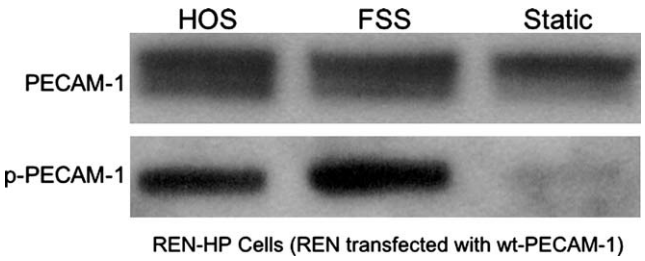

Fig. 4. HOS and 13 dyn/cm^2 FSS lead to tyrosine phosphorylation of PECAM-1 in REN-HP cells. Cell lysates were immunoprecipitated with anti-PECAM-1 mAb 4G6 and membranes were blotted with anti-PECAM-1 mAb 1.3 (upper panel), stripped and reprobed with anti-PY pAb PY20 (lower panel).

in REN-HP cells when compared with static control (Fig. 4). In Western blotting of lysates of REN cells, a negative control lacking PECAM-1, as expected, no corresponding band was visible (data not shown).

Homophilic PECAM-1 binding is not required for mechanically induced tyrosine phosphorylation

In a study of mechanically induced PECAM-1–tyr-P, Osawa et al. [6] proposed a model in which mechanical force acts directly on PECAM-1, causing a conformational change that permits tyr-P of the cytoplasmic domain of PECAM-1. To explore whether lateral cell–cell adhesion site localization and homophilic binding between PECAM-1 molecules on adjacent cells are required for force-induced PECAM-1–tyr-P, REN cells stably expressing the K151/R152A mutant form of PECAM-1 were exposed to 15 min of FSS or 10 min of HOS. In previous work, we noted that this mutant form of PECAM-1 does not localize to the lateral cell–cell border (Fig. 2) and does not support homophilic binding [15]. FSS and HOS stimulated increased PECAM-1–tyr-P in REN cells transfected with the K151/R152A mutant form of PECAM-1 (Fig. 5), demonstrating that membrane localization and homophilic binding between confluent cells are not required for mechanosignaling responses.

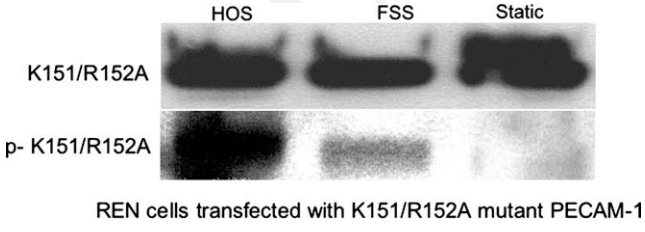

Fig. 5. HOS and 13 dyn/cm^2 FSS induce tyrosine phosphorylation of PECAM-1 in REN cells transfected with the K151/R152A mutant of PECAM-1. Cell lysates were immunoprecipitated with anti-PECAM-1 mAb 4G6 and membranes were blotted with anti-PECAM-1 pAb PCD (upper panel), stripped, and reprobed with anti-PY pAb PY20 (lower panel).

The extracellular and transmembrane domains of PECAM-1 are not required for mechanically induced tyrosine phosphorylation

To explore the importance of the extracellular and transmembrane domains of PECAM-1 in mechanosensation, we exposed REN cells transfected with mutant forms of PECAM-1 to FSS. In previous experiments, we have demonstrated that when a mutant form of PECAM-1 containing the non-homologous IL2R extracellular and transmembrane domains fused to the intact PECAM-1 cytoplasmic domain (IL2PCD mutant) is transfected into REN cells, it is expressed diffusely throughout the cell membrane (Fig. 2), but continues to serve as a substrate for c-Src-dependent, H_2O_2-induced PECAM-1–tyr-P [15,20]. In addition, this PECAM-1 mutant regulates H_2O_2-induced cation channel activity with kinetics identical to that of wt-PECAM-1 [20]. REN cells stably transfected with the IL2PCD mutant of PECAM-1 were exposed to FSS and HOS as described above in the presence of phosphatase inhibition by vanadate. Both forms of mechanical stress resulted in increased PECAM-1–tyr-P in REN cells transfected with the IL2PCD mutant form of PECAM-1 (Fig. 6).

After confirming that native PECAM-1 undergoes tyrosine phosphorylation in response to mechanical stress in human endothelial cells, we have reproduced the phenomenon in endothelium-like REN cells transfected with PECAM-1. Since this is a null cell, it permitted an investigation of altered protein structure and cellular localization in PECAM mechanosensing by transfection of PECAM-1 mutant constructs. Lateral cell–cell border localization is not required for force-induced PECAM-1 tyrosine phosphorylation. Osawa et al. [6] demonstrated a similar finding in sparsely cultured cells. Our work extends this finding to the highly structured confluent monolayer, a situation found in vivo and reproduced in both endothelial and REN in vitro, where homophilic binding occurs between PECAM-1 molecules on adjacent cells. Not only does force-induced PECAM-1–tyr-P appear to be independent of PECAM localization to the lateral membrane, but it appears that neither the extracellular nor transmembrane domains are necessary for mechanosignaling. The mutant forms of PECAM-1–tyr-P also associated with the phosphatase SHP-2, as shown by other investigators [19] indicating all transfected forms of PECAM-1 to be a substrate for a tyrosine kinase in the present study. The evidence suggests that mechanosensors may activate a tyrosine kinase that in turn phosphorylates the cytoplasmic domain of PECAM-1, leading to SHP2 activation and eventually Erk-1/2 activation. It has been suggested that PECAM-1 may regulate or associate with other potentially mechanoresponsive molecules: β-catenin [21], focal adhesion kinase [22], and integrin $\alpha_V\beta_3$ [23,24]. Whether the mechanically induced behavior of

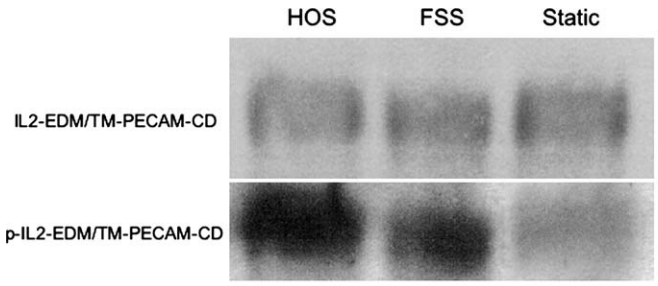

Fig. 6. HOS and 13 dyn/cm2 FSS lead to tyrosine phosphorylation of PECAM-1 in REN cells transfected with the IL2PCD mutant of PECAM-1. Cell lysates were immunoprecipitated with anti-IL2R mAb and membranes were blotted with anti-PY pAb PY20 (lower panel). Membranes were then stripped and reprobed with anti-PECAM-1 pAb PCD (upper panel).

these molecules modulates or is modulated by PECAM-1 has not been elucidated.

Mechanical stress is an important determinant of endothelial cell behavior [1,2]. Areas of disturbed flow, for example, are more prone to atheroma formation [25,26]. Abnormal mechanical stress may also play a role in the pathogenesis of pulmonary hypertension [27], ventilator-induced lung injury [28,29] or glomerulonephropathy [30]. PECAM-1 is expressed abundantly on endothelial cells, platelets, and most leukocytes. It is believed to play a role in mediating adhesion between adjacent endothelial cells, angiogenesis, and neutrophil adhesion to, and migration through, the endothelial monolayer [9,31,32]. PECAM-1-null mice, however, do not display developmental abnormalities or significant vascular defects, although bleeding time is increased, leukocyte transendothelial migration is slowed, and the blood–brain barrier may be weakened [33–35].

In summary, utilizing EC-like REN cells stably transfected with wild-type and mutant PECAM-1 constructs to elucidate which domains of PECAM-1 confer mechanosensitivity, we demonstrate that in the confluent monolayer, phosphorylation does not depend upon lateral membrane localization of the protein and cell–cell homophilic PECAM-1 binding. The transmembrane and extracellular domains of PECAM-1 are not necessary for mechano-responsiveness. The kinase, which remains to be identified, appears to be activated by a more direct effect of mechanical stress on the cells.

Acknowledgments

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