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Phosphorylation of Leukocyte PECAM and Its Association with Detergent-Resistant Membranes Regulate Transendothelial Migration

Oliver Florey,*,† Joanne Durgan,‡ and William Muller*

Leukocyte migration across the endothelial lining is a critical step in the immune response (1). This process is tightly regulated by a number of proteins to ensure migration to the proper location at the appropriate time. PECAM is a transmembrane protein belonging to the Ig superfamily (2). It is constitutively expressed in both endothelial cells and leukocytes and plays a critical role in the process of leukocyte transendothelial migration (3–5). Specifically, it is the homophilic interaction between endothelial PECAM and leukocyte PECAM that is required for transmigration. To date, most mechanistic studies of the role of PECAM in transmigration have focused on the endothelial side (6–8).

Leukocyte PECAM also plays a critical role during transmigration (5). However, its precise role is not well understood. It may serve primarily as an adhesion molecule or, like its endothelial counterpart, actively engage in signal transduction events that drive transmigration. To test this hypothesis, we have used Ab cross-linking methods to manipulate the timing of leukocyte PECAM activation during transmigration. In this study, we provide evidence that PECAM ligation leads to the activation of leukocyte signaling pathways that are critical during transmigration.

The cytoplasmic tail of PECAM contains tyrosine residues at positions 663 and 686, which constitute ITIMs (9, 10). Src kinases have been shown to phosphorylate these residues (11, 12), which then serve as docking sites for Src homology 2 domain-containing proteins such as Src homology region 2 domain-containing phosphatase (SHP) 1, SHP2, phospholipase Cγ, and SHIP (13–15). It is through these phosphorylation events and subsequent protein interactions that PECAM can mediate downstream signaling. Although the requirement for endothelial PECAM phosphorylation has been well studied with respect to endothelial junction function (16, 17) and leukocyte transmigration (6), the role of leukocyte PECAM phosphorylation in the transmigration process remains uncharacterized. In the current study, we generated a leukocyte cell line in which endogenous PECAM was depleted and replaced with either wild-type or nonphosphorylatable Y663F/Y686F mutants. Using this approach, we demonstrate that the phosphorylation of leukocyte PECAM is also required for transmigration.

Several immunoreceptors, including TCR, BCR, and FcγRs, initiate signal transduction by associating with specialized lipid regions in the membrane (18–20), termed detergent-resistant membranes (DRMs) (21). PECAM has previously been shown to associate with DRMs in platelets (22), but this association has not been studied in leukocytes. We present evidence that leukocyte PECAM moves into DRMs during transmigration, where is displays reduced phosphorylation. Forcing PECAM into DRMs decreases transmigration. The data are consistent with a model in which homophilic interaction of PECAM induces signaling through PECAM phosphorylation that is necessary for transmigration. The activation of PECAM is then terminated by movement of PECAM into DRMs.

Materials and Methods

Abs and reagents

Monoclonal mouse anti-human hec7 (anti-PECAM) (23) and hec2 (anti-CD99) (24) were produced from hybridomas. Polyclonal rabbit anti-human PECAM 177 and 301 were generated in-house. The nonblocking mouse...
anti-PECAM mAb P1.1 was a kind gift from Dr. Peter Newman (Blood Center of Wisconsin, Milwaukee, WI). Anti-phytohemagglutinin 4G10 was purchased from Millipore (Bedford, MA). F(ab')2 goat anti-mouse and goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit anti-mouse IgG-HRP and swine anti-rabbit IgG-HRP were purchased from DakoCytomation (Carpinteria, CA). Src kinase inhibitor PP2 was purchased from Calbiochem (San Diego, CA). Methyl-β-cyclodextrin (MβCDX) was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and differentiation

U937L cells (kindly donated by Dr. F. William Luscinkas, Brigham and Women’s Hospital, Boston, MA) were maintained in RPMI 1640, 10% FBS, t-glutamine, and penicillin/streptomycin and differentiated to a more monocyte lineage with 1 mM dithyryl-CAMP (Sigma-Aldrich) stimulation for 3 d. These cells have previously been demonstrated to transmigrate HUVEC monolayers in a similar manner to primary monocytes (25). HUVECs were isolated from fresh umbilical cords, as previously described (23), and grown in medium 199 (M199, Invitrogen, Carlsbad, CA) supplemented with 20% adult human serum and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2. Experiments were done routinely with cells at passage two plated on thick hydrated type I collagen gels in 96-well culture plates.

PBMCs and monocyte isolation

PBMCs were isolated from healthy volunteers by density gradient centrifugation in Ficoll-Paque. Monocytes were isolated from the PBMC fraction using an MACS monocyte isolation kit and magnetic depletion columns according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). This yielded >90% monocytes, as determined by flow cytometry following labeling with FITC-labeled anti-CD14.

Generation of U937L PECAM knockdown cell line

A GFP-tagged lentiviral short hairpin RNA (shRNA) construct against human PECAM (PEC02) and a nonsilencing control (NSC) construct were generously donated by Dr. Peter Newman and have been previously described (26). U937L cells were infected with lentiviral constructs at a multiplicity of infection of 100 for 5 h at 37°C. Cells, designated U937L PEC02 or U937L NSC were washed and replated in a 12-well plate in 1 ml normal culture media. After 3 d, strongly GFP-positive cells were selected using an FACSVantage cell sorter (BD Biosciences, San Jose, CA).

Lentiviral PECAM rescue construct and transduction of U937L cells

Full-length human PECAM in the lentiviral plasmid pWPT (kindly donated by Dr. Peter Newman) was used as a template to make PECAM rescue constructs. Six silent mutations within the PECAM small interfering RNA target region were introduced using site-directed mutagenesis (Stratagene, La Jolla, CA) along with tyrosine and phenylalanine mutations at positions 663 and 686, as described in the Supplemental Material. pWPT PECAM constructs were mixed with the packaging plasmid PMCVR8.2 and envelope vector pVSV-G and cotransfected into 293FT cells. Lentiviral particles were purified, and U937L PEC02 cells were transduced to achieve expression levels close to those of endogenous PECAM as verified by flow cytometry.

Western blotting

Cells were lysed for 10 min at 4°C in nondenaturing lysis buffer A, 1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM NaVO3, 1 mM PMSF, and protease inhibitor mixture (Sigma-Aldrich). Samples were then spun at 14,000 rpm for 10 min at 4°C. Supernatants (Triton soluble fraction) were collected, and the remaining pellets (Triton insoluble) were resuspended in sample buffer and boiled for 5 min, passed through a 26-gauge needle five times, sonicated for 5 min, and sonicated for 5 min. Samples were then diluted 1:10 with nondenaturing lysis buffer before being added to Protein G beads as above.

Isolation of DRMs by sucrose density gradient ultracentrifugation

This assay was performed and quantified as previously described (5). In brief, PBMCs or differentiated U937L cells were preincubated or without 10 μg/ml anti-PECAM Abs on ice for 15 min washed and resuspended in M199 plus 0.1% HSA at 2 × 10^6/ml. A total of 100 μl cells were added to each replicate well of confluent monolayers of HUVECs grown on hydrated collagen gels and incubated for 1 h at 37°C in a CO2 incubator. To induce surface leukocyte PECAM cross-linking, F(ab')2 secondary Abs, 50 μg/ml final concentration, were added to the desired wells. The percentage of transmigration in 10 different fields of view was calculated by dividing the number of leukocytes below the HUVEC monolayer by the total number of leukocytes in the field.

Fluorescent microscopy on Triton-extracted samples

Confluent HUVEC monolayers were grown on fibronectin-coated glass-bottomed dishes and prelabeled with 20 μg/ml Alexa Fluor 488-conjugated anti–vascular endothelial (VE)-cadherin for 20 min at 37°C. PBMCs were prelabeled with Alexa Fluor 546-conjugated nonblocking anti-PECAM mAb (P1.1) on ice before washing and resuspension in M199 plus 0.1% HSA at 2 × 10^6/ml. A total of 100 μl PBMCs was added to monolayers of HUVECs for 10 min at 37°C. Dishes were either fixed in 2% paraformaldehyde or treated with cold Triton X-100 extraction buffer (0.5% Triton, 138 mM KCl, 3 mM MgCl2, 2 mM EDTA, 0.32 M sucrose, 10 mM MES, protease inhibitors, 1 mM PMSF [pH 6.1]) for 20 min on ice prefixation. Dishes were imaged using a Deltavision microscope (Applied Precision, Issaquah, WA). Analysis of fluorescent intensity was carried out using Metamorph software (Analytical Technologies, Sunnyvale, CA).

Statistics

The Student t test was used to evaluate statistical significance in the data presented using Prism (GraphPad, San Diego, CA).

Results

Cross-linking monocyte PECAM promotes transendothelial migration

Leukocyte transendothelial migration can be inhibited using anti-PECAM neutralizing Abs (4, 5) that bind to the homophilic interaction domain, preventing leukocyte PECAM interaction with endothelial cell PECAM. If these neutralizing Abs are subsequently clustered by cross-linking with a secondary Ab, PECAM signaling can be artificially activated. This system allows us to mimic the concentrated PECAM ligation that occurs in time and space on a leukocyte during transmigration and to temporally control PECAM activation in the treated cells.

To analyze the role of leukocyte PECAM, we added primary monocytes, treated with a blocking anti-PECAM IgG Ab, to endothelial monolayers for 30 min. This allowed sufficient time for monocytes to migrate to endothelial junctions, where they are arrested (Fig. 1). Clustering of leukocyte PECAM to induce its activation was then performed by the addition of a F(ab')2 secondary cross-linking Ab after 30 min. Fig. 1 shows that blocking monocyte PECAM with mAb hec7 inhibited transmigration. Strikingly, cross-linking PECAM on these arrested cells, after 30 min,
were not inhibited by blocking FcRs, as we see similar results using F(ab')2 Abs, and effects were not inhibited by blocking FcγRs (data not shown). These data suggest that the role of leukocyte PECAM extends beyond its function as an adhesion receptor and that activation of this molecule may be required during transmigration. We found no evidence to suggest that PECAM activation mediates the release of soluble factors that aide the transmigration event (Supplemental Fig. 1), although we cannot formally exclude the possibility of extremely localized short-lived factors.

**Leukocyte PECAM is phosphorylated upon cross-linking**

Next, we investigated the possibility that leukocyte PECAM phosphorylation is involved in transmigration by engaging in intracellular signaling transduction. PECAM contains several tyrosine residues that may become phosphorylated upon activation; the best characterized of these are at positions 663 and 686, which reside within ITIM-like domains. PECAM is not phosphorylated in resting monocytes, but becomes rapidly and transiently tyrosine phosphorylated upon cross-linking (Fig. 2A). Using dibuteryl cAMP-differentiated U937L cells as a monocyte model, we were able to see the same pattern of transient PECAM phosphorylation after cross-linking (Fig. 2B). The phosphorylation of PECAM was inhibited using the Src kinase inhibitor PP2 (Fig. 2C). Results shown are representative of three separate experiments. IB, Western blotting; IP, immunoprecipitation.

**Phosphorylation of tyrosine 663/686 on leukocyte PECAM is required for transendothelial migration**

To test whether leukocyte PECAM phosphorylation is involved in transendothelial migration, we generated monocytic U937L cell lines expressing either wild-type or mutant forms of PECAM lacking the 663 and 686 tyrosine phosphorylation sites. To make sure that the cells expressed the same levels of total PECAM, we first measured the phosphorylation status of re-expressed PECAM at close to endogenous levels. The phosphorylation status of re-expressed PECAM was tested by immunoprecipitation and Western blotting (Fig. 3A). These data clearly show that U937L cell transmigration is dependent upon leukocyte PECAM.

**Discussion**

These results substantiate our previous findings and provide new insights into the role of leukocyte PECAM in transmigration. The phosphorylation events triggered by leukocyte PECAM clustering were involved in the transmigration process. Leukocyte PECAM becomes tyrosine-phosphorylated after cross-linking. Human monocytes (A) or differentiated U937L cells (B) were stimulated by PECAM cross-linking for the indicated times and lysates were immunoprecipitated with anti-PECAM Abs. Samples were resolved on SDS-PAGE gels and analyzed by Western blotting (IB) using anti-phosphotyrosine and PECAM Abs. Band intensities were measured via densitometry, and the ratio of phosphorylated PECAM to total PECAM is shown below the Western blots. C, Differentiated U937L cells were left either untreated or pretreated with the Src kinase inhibitor PP2 (20 or 100 μM, 30 min) before PECAM cross-linking for 3 min. Lysates were analyzed by Western blotting (performed as in A and B). Results shown are representative of three separate experiments. IB, Western blotting; IP, immunoprecipitation; US, unstimulated (no PECAM cross-linking).

**Figures**

**FIGURE 1.** Cross-linking leukocyte PECAM reverses an anti-PECAM block in transmigration. Human monocyte transmigration through unstimulated HUVEC monolayers grown on collagen gels. Monocytes were prelabeled on ice with blocking Abs against PECAM (hec7, 177) and CD99 (hec2) at 20 μg/ml before being washed free of unbound Abs and added to HUVEC monolayers for 60 min. PECAM was activated by the addition of cross-linking secondary Abs (50 μg/ml) 30 min into the assay where indicated. Data represent mean ± SEM from seven separate experiments. **p < 0.003; ***p < 0.001.

**FIGURE 2.** Leukocyte PECAM becomes tyrosine-phosphorylated after cross-linking. Human monocytes (A) or differentiated U937L cells (B) were stimulated by PECAM cross-linking for the indicated times and lysates were immunoprecipitated with anti-PECAM Abs. Samples were resolved on SDS-PAGE gels and analyzed by Western blotting (IB) using anti-phosphotyrosine and PECAM Abs. Band intensities were measured via densitometry, and the ratio of phosphorylated PECAM to total PECAM is shown below the Western blots. C, Differentiated U937L cells were left either untreated or pretreated with the Src kinase inhibitor PP2 (20 or 100 μM, 30 min) before PECAM cross-linking for 3 min. Lysates were analyzed by Western blotting (performed as in A and B). Results shown are representative of three separate experiments. IB, Western blotting; IP, immunoprecipitation; US, unstimulated (no PECAM cross-linking).
PECAM cross-linking (Fig. 4A), which are known to associate with Triton X-100–insoluble fractions and remain in the same fractions before and after PECAM cross-linking. This movement to an insoluble fraction was specific to PECAM, as other receptors, CD64 and CD32a, were not moved into DRMs or association with the cytoskeleton. We found that movement of PECAM to an insoluble fraction were seen upon cross-linking (Fig. 4B). Following PECAM cross-linking for 3 min, lysates from all the U937L cell lines were immunoprecipitated with anti-PECAM and probed for anti-phosphotyrosine. Ratios of phospho-PECAM in the cell lines are presented below the blots. From these results, we conclude that the tyrosine phosphorylation within the ITIM domains of leukocyte PECAM plays a critical role in transendothelial migration.

Leukocyte PECAM resists detergent extraction after cross-linking

Coupled with the observation of leukocyte PECAM phosphorylation after cross-linking, a change in its detergent solubility was also detected. Fig. 4A shows that U937L PECAM is completely solubilized in 1% cold Triton X-100 lysis buffer under resting conditions. However, following 3 min of PECAM cross-linking, ~50% of PECAM is found in the insoluble pellet. Movement of PECAM, as other receptors, CD64 and CD32a, which are known to associate with Triton X-100-insoluble membranes upon activation, remain in the soluble fraction after PECAM cross-linking (Fig. 4A).

Resistance to cold Triton extraction could be due to partitioning into DRMs or association with the cytoskeleton. We found that U937L PECAM could be efficiently solubilized using warm 1% Triton X-100 lysis buffer even after cross-linking, a property that would be inconsistent with cytoskeletal association (data not shown). Similarly, PECAM insolvency was not due to cytoskeleton association, as cytochalasin D had no effect on cross-linking–induced PECAM movement to DRMs (data not shown). These results are therefore consistent with PECAM partitioning into DRMs. Furthermore, we found that the movement to an insoluble fraction is both rapid and prolonged. PECAM translocated to DRMs within 3 min and remained there for up to 60 min after cross-linking (Fig. 4B).

**PECAM is recruited to low-density DRMs after its cross-linking**

To further test the hypothesis that PECAM was recruited to DRMs after cross-linking, we employed a more definitive technique. DRMs can be purified by density ultracentrifugation due to their high lipid content. After cross-linking PECAM, differentiated U937L cells were subjected to sucrose gradient centrifugation. Fig. 4C shows that, in unstimulated cells, PECAM was found in the high-density fractions of the gradient representing the soluble protein. After its cross-linking, movement of a portion of PECAM to lower density fractions was observed. A known DRM component, lyn kinase (27), was also enriched in the same light-density fraction and remained the same before and after PECAM cross-linking.

Depletion of membrane cholesterol by MβCD has previously been shown to disrupt DRMs (28). Pretreating cells with 20 mM MβCD for 45 min inhibited PECAM translocation to DRMs upon cross-linking (Fig. 4C). Lyn kinase also moved to a cold Triton-soluble fraction after MβCD treatment, verifying the disruption of DRMs. Similar results of cholesterol-dependent movement of PECAM to a cold Triton-insoluble fraction were seen using purified human monocytes (Fig. 4D). These cells remained...
viable after MβCDX treatment and showed clear signs of cholesterol depletion, including cell rounding and loss of polarized morphology (data not shown). The movement of PECAM to DRMs is independent of tyrosine phosphorylation, as PP2 did not stop PECAM movement to DRMs after cross-linking. PECAM from the P-663/686 cell line also translocates to DRMs after cross-linking (Supplemental Fig. 2).

**Leukocyte PECAM is found in DRMs during transendothelial migration**

We next asked whether movement of PECAM into DRMs actually occurs during leukocyte transmigration. To do this, we designed an assay to detect Triton X-100-insoluble proteins using detergent extraction and fluorescence microscopy. In this assay, monocytes were prelabeled with an Alexa Fluor 546-conjugated anti-PECAM Ab (P1.1), which does not block transmigration. Endothelial cells were preincubated with a nonblocking Alexa Fluor 488-conjugated anti–VE-cadherin to mark junctions. Fig. 5A shows that PECAM of unstimulated monocytes adhered to glass is completely removed from cells by cold Triton X-100 extraction, whereas VE-cadherin remains visible at endothelial cell junctions. Some PECAM staining was still visible away from the junction showed no PECAM staining (asterisk) in cold Triton buffer. Soluble and insoluble fractions in the supernatant and insoluble proteins in the pellet were analyzed by Western blotting using Abs against PECAM, CD64, CD32, and actin. Quantification of levels of PECAM from each fraction is shown below; data are from seven separate experiments. B, Soluble and insoluble fractions from U937L cells stimulated for increasing times by PECAM cross-linking were probed for PECAM. C, U937L cells were left untreated or pretreated with 20 μM MβCDX for 45 min before undergoing PECAM cross-linking for 3 min. Lysates were run over a sucrose density gradient and fractions probed for PECAM or Lyn kinase by Western blotting. D, Purified human monocytes with or without MβCDX pretreatment were stimulated by PECAM cross-linking for 3 min followed by lysis in cold Triton buffer. Soluble and insoluble fractions were analyzed by Western blotting using Abs against PECAM. I, insoluble; IB, Western blotting; S, soluble.

**Tyrosine-phosphorylated leukocyte PECAM is enriched in non-DRM regions**

As the phosphorylation state of PECAM was not important in its movement to DRMs (Supplemental Fig. 2), we investigated whether the inclusion of PECAM in DRMs had an effect on PECAM phosphorylation. Unexpectedly, we found that phosphorylated PECAM in U937L cells was more abundant in the Triton-soluble fraction than the insoluble fraction (Fig. 6). The levels of PECAM protein appear lower in insoluble fractions; however, this is a reflection of the efficiency of immunoprecipitation from insoluble fractions compared with soluble fractions rather than a difference in total protein. Thus, the PECAM to phospho-PECAM ratio was used to compare the fractions. Consistent with this result, disruption of DRMs by MβCDX treatment resulted in a hyperphosphorylation of PECAM. These data suggest that PECAM is phosphorylated in non-DRM regions, whereas PECAM in DRMs either undergoes less phosphorylation or is actively dephosphorylated.

**Leukocyte PECAM movement to DRMs negatively regulates transendothelial migration**

We have demonstrated that leukocyte PECAM phosphorylation is important for transmigration and also that leukocyte PECAM in DRMs is less phosphorylated after cross-linking. We next sought to examine whether movement of leukocyte PECAM to DRMs affected transendothelial migration. Transmigration experiments using MβCDX to disrupt DRMs could not be performed, as cholesterol depletion affects the ability of leukocytes to adhere, polarize, and migrate, which would all impair the assay (29–31). Instead of inhibiting PECAM movement, we thus chose to force and lock PECAM into DRMs. To achieve this without affecting PECAM’s ability to undergo homophilic interactions, we exploited
a nonblocking anti-PECAM Ab (P1.1), which does not inhibit transmigration, but when cross-linked translocates PECAM to DRMs and retains it there (Fig. 7A) without affecting its homophilic adhesion properties (Supplemental Fig. 3). Forcing leukocyte PECAMs into DRMs through P1.1 cross-linking prior to their addition to HUVEC (t = 0) resulted in profound inhibition of transmigration. Meanwhile, P1.1 on its own or cross-linked after 30 min (t = 30) had little effect (Fig. 7B). Similarly, cross-linking PECAM with the blocking Ab hec7 at t = 0 inhibited transmigration, whereas cross-linking at t = 30 overcomes the block (Figs. 1, 7C). These data suggest that the timing of leukocyte PECAM activation and subsequent translocation to DRMs is important for transmigration. Forcing PECAM into DRMs before monocytes are able to get to endothelial junctions (i.e., at t=0) inhibits transendothelial migration. Together, these data suggest that the retention of leukocyte PECAM in DRMs leads to a decrease in its phosphorylation and a subsequent inhibition of transmigration.

**Discussion**

PECAM has previously been implicated in signal transduction mediated by the phosphorylation of its cytoplasmic tail and association with other signaling complexes (32). Because PECAM is so fundamental to the process of leukocyte transmigration, it would seem likely that it acts as a signaling molecule in this setting, too. Although much work has been undertaken to elucidate the role of endothelial PECAM, far less has been done to explore the function of leukocyte PECAM during transmigration. Our present study reveals a key role for the tyrosine phosphorylation of leukocyte PECAM in transmigration. Furthermore, we describe a novel mechanism regulating the phosphorylation of leukocyte PECAM through its association with specialized lipid domains (DRMs).

Many immune receptors have been shown to cluster on the cell surface after ligand binding, and this clustering plays an important role in initiating downstream signaling events (33). There is a high local enrichment of endothelial PECAM at junctions, and it seems likely that this may induce leukocyte PECAM to cluster during transmigration. We hypothesized that such a clustering of leukocyte PECAM might transmit signals required for the continuation and completion of transmigration. Ab mediated cross-linking of surface receptors initiates signal transduction, although the actual mechanism of how this works remains unclear. In the current study, we used cross-linking of leukocyte PECAM in a novel manner to mimic its natural clustering and thus precisely control its activation during transmigration.

Blocking leukocytes with anti-PECAM Abs inhibited homophilic engagement with endothelial cell PECAM and thus arrested cells at endothelial junctions, unable to transmigrate through the monolayer (5, 34). Importantly, we found that the subsequent activation of leukocyte PECAM by the addition of secondary cross-linking Abs was sufficient to promote transmigration in a subpopulation of the arrested cells. We hypothesize that the partial nature of the recovery may reflect the fact that only leukocytes located at permissible sites (e.g., endothelial junctions) at the time of cross-linking would be prompted to transmigrate. For those cells not engaging endothelial junctions at the time, PECAM cross-linking would drive PECAM into DRMs, where it would be held in a state of deactivation that would actually inhibit transmigration. Indeed, cross-linking PECAM on leukocytes before they get to endothelial junctions inhibits their transmigration (Fig. 7). These data confirm that leukocyte PECAM...
is critical during transmigration and suggest that, in addition to its accepted role as an adhesion molecule, it plays an active signaling function.

As phosphorylation was a consequence of PECAM ligation, we examined its role in transmigration using dibutyl cAMP-differentiated U937L cells in which we depleted endogenous PECAM and re-expressed phospho-mutant constructs. We found that depletion of endogenous PECAM led to a block in transmigration that could be rescued by re-expression of the wild-type protein. Strikingly however, U937L cells re-expressing PECAM with tyrosine-phosphorylated sites 663 and 686 displayed a susceptibility that could be rescued by re-expression of the wild-type protein. The subsequent phosphorylation of leukocyte PECAM drives as yet unknown mechanisms required for the continuation and completion of the transmigration process. After PECAM engagement, its phosphorylation and signaling in the leukocyte membrane are terminated by translation to DRMs. ∗∗∗∗∗ < 0.02; ∗∗∗∗ < 0.002. IB, Western blotting.

Previous studies suggest that a downstream consequence of PECAM phosphorylation at sites 663 and 686 is the recruitment and activation of Src homology 2 domain-containing phosphatases SHP1 and SHP2 (13–15). Other signaling proteins, such as phospholipase Cγ and SHIP, have also been proposed to complex with phosphorylated PECAM, although this remains controversial (35, 36), and indirect association of P3K and Grb2 with PECAM has been reported (15, 37). PECAM is often cited as a negative regulator of signaling (38–0), but SHP2 is capable of transmitting stimulatory signals (41, 42). The fact that we see an increase in transmigration upon PECAM ligation suggests that, if in our system, PECAM acts as a stimulatory molecule. Consistent with this possibility, we and other groups have reported integrin activation downstream of leukocyte PECAM ligation (34, 43, 44). The identification and characterization of signals initiated downstream of leukocyte PECAM phosphorylation is currently under investigation. It is tempting to speculate that phosphorylation of leukocyte PECAM at endothelial junctions may activate a subpopulation of integrins to provide an anchor point while the cell passes through the endothelial monolayer. Integrin affinity changes have been monitored during leukocyte migration over endothelial substrates (45), but as yet there are no studies looking at their activation status during transmigration.

The association of immune receptors with specialized domains within the plasma membrane has been implicated in their ability to transduce signals (33). The clustering of receptors into these specialized lipid domains brings them into close proximity with and exposes them to other proteins, such as kinases, phosphatases, and scaffolding proteins that act to initiate and amplify signal transduction. These domains are often rich in cholesterol and sphingolipids, which reduces their solubility in some nonionic detergents (21, 46), and are often termed DRMs. We provide evidence that in resting monocytes, PECAM is found almost exclusively in a Triton X-100–soluble fraction, but that a portion of it becomes associated with DRMs after cross-linking. We examined PECAM movement to DRMs both by analyzing its solubility in different lysis buffers and by sucrose gradient analysis. Using both techniques, we obtained clear evidence for this activation-induced translocation, although the percentage of PECAM shifted differed. This disparity is most likely due to differences in the methods used to obtain samples and the efficiency of solubilization during the procedures. Although we cannot accurately define the exact amount of PECAM that moves to the DRM, we were able to efficiently manipulate the translocation and analyze its functional implications.

Most important, we found that leukocyte PECAM associates with DRMs during actual transmigration (Fig. 5). PECAM on leukocytes adherent to endothelial monolayers demonstrated greater detergent

![FIGURE 7. Activating PECAM prior to adhesion inhibits transmigration. A, Monocytes were stimulated by PECAM cross-linking using the monoclonal antibody P1.1 for the indicated times. Triton X-100 soluble and insoluble fractions were analyzed by Western blotting. Human monocyte transmigration through unstimulated HUVEC monolayers grown on collagen gels. Monocytes were prelabeled on ice with non-blocking Ab against PECAM (P1.1) (B) or blocking Ab against PECAM (Hec7) (C) at 20 μg/ml before being washed free of unbound Ab and added to HUVEC monolayers for 60 min. Cross-linking secondary Abs (50 μg/ml) were added at the start of the assay (t = 0) where indicated. Data represent mean ± SEM from three separate experiments. D, Proposed model of leukocyte PECAM regulation during transendothelial migration. Leukocytes migrate to endothelial cell junctions where they homophilically engage PECAM. The subsequent phosphorylation of leukocyte PECAM drives as yet unknown mechanisms required for the continuation and completion of the transmigration process. After PECAM engagement, its phosphorylation and signaling in the leukocyte membrane are terminated by translation to DRMs. ∗∗∗ < 0.02; ∗∗∗∗ < 0.002. IB, Western blotting.](http://www.jimmunol.org/Downloadedfrom)
resistance when located at endothelial junctions compared with positions away from junctions. PECAM ligation and clustering would likely occur at junctions due to the local enrichment of endothelial PECAM. Amplification of leukocyte PECAM clustering in this region could be achieved by the active enrichment of endothelial PECAM from the lateral border-recycling compartment that occurs in transmigration(7,47). Ours is the first direct evidence to suggest that leukocyte proteins partition into DRMs during the transmigration process. It would be of interest to see if other leukocyte proteins known to be involved in transmigration also partition into DRMs.

For most transmembrane receptors, movement into DRMs is associated with an increase in their signaling potential. This can be due to the exclusion of negative regulators from the DRM, such as phosphatases (48), or the enrichment of activating kinases (49, 50). Unexpectedly, we found that PECAM association with DRMs had a negative effect on its activation and signaling capacity; PECAM was less phosphorylated in the Triton-insoluble fraction as compared with soluble fractions. Consistent with this, we saw that disruption of DRMs by cholesterol depletion led to hyper-phosphorylation of PECAM phosphorylation within the Triton-insoluble fraction. As the kinase and/or phosphatase involved in controlling leukocyte PECAM phosphorylation are not known, it is difficult to understand by what mechanism PECAM is negatively regulated in DRMs.

Because PECAM association with DRMs decreases its phosphorylation (Fig. 6), and our data (Fig. 3) define a key role for leukocyte PECAM phosphorylation in transmigration, we sought to determine if the movement of PECAM to DRMs affected transmigration. Experiments to disrupt DRMs via cholesterol depletion could not be carried out in the context of transmigration, as this disrupts many crucial upstream processes (i.e., adhesion, polarization, migration). Instead, we chose to force PECAM into DRMs by cross-linking with nonblocking anti-PECAM Abs prior to incubation on the endothelial monolayer. This does not affect its ability to homophilically bind PECAM but could potentially affect its ability to initiate further signal transduction. Cross-linking PECAM on leukocytes this way, before they have adhered and migrated to endothelial junctions, significantly inhibits their ability to transmigrate (Fig. 7). We propose that this is due to cross-linked PECAM being shifted to DRMs, where its phosphorylation capacity is lower. These data may seem at odds with that of Fig. 1, in which cross-linking PECAM on blocked leukocytes promoted transmigration. However, a critically important distinction is that PECAM activation was induced at different times in these experiments. When leukocyte PECAM is activated at the start of the assay, before leukocytes adhere or move to the junctions, there is no transmigration because PECAM has been sequestered in DRMs in a long-term, dephosphorylated, signaling-incompetent state. However, when cross-linking is performed when cells are poised at junctions ready to transmigrate, transmigration is promoted because the transient activation of PECAM can drive this process. Our current working model proposes that during normal transmigration, leukocyte PECAM is subject to transient activation, stimulated by engagement with locally enriched endothelial PECAM at the junction, which is critical to drive transmigration. This may be followed shortly afterward by downregulation of PECAM signaling by sequestration and dephosphorylation in DRMs (Fig. 7D). This may turn off the local signaling within the leukocyte at that point, allowing leukocyte deadhesion and progression of that portion of the leukocyte across the endothelial cell border. All of this is likely to work in concert with PECAM-mediated events triggered in the endothelial cell.

In summary, we provide evidence that the precise spatial and temporal control of leukocyte PECAM signaling is required for transmigration. Deregulation of leukocyte PECAM activation disrupts transmigration. Further work will be required to define the signaling events downstream of leukocyte PECAM and to establish whether dysregulation of this pathway may contribute to disease.

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Disclosures

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References


