Migration of Monocytes Across Endothelium and Passage through Extracellular Matrix Involve Separate Molecular Domains of PECAM-1

By Fang Liao, Hanh K. Huynh, Ana Eiroa, Tricia Greene, Elizabeth Polizzi, and William A. Muller

Summary

During the inflammatory response, the adhesion molecule PECAM plays a crucial role in transendothelial migration, the passage of leukocytes across endothelium. We report here an additional role for PECAM in the subsequent migration of monocytes through the subendothelial extracellular matrix. PECAM has six immunoglobulin (Ig) superfamily domains. Monoclonal antibodies whose epitopes map to domains 1 and/or 2 selectively block monocyte migration through the endothelial junction, whereas those that map to domain 6 block only the migration through the extracellular matrix, trapping the monocyte between the endothelium and its basal lamina. Therefore, transendothelial migration (diapedesis) and passage through extracellular matrix (interstitial migration) are distinct and separable phases of monocyte emigration. Furthermore, distinct and separate Ig domains of PECAM are involved in mediating these two steps.

During the final stages of emigration from the bloodstream into a site of inflammation, white blood cells, or leukocytes, squeeze between tightly apposed endothelial cells (EC) of the vascular wall without disrupting vascular permeability. This transendothelial migration (TEM) involves platelet/endothelial cell adhesion molecule-1 (PECAM, CD31), which is expressed on the surfaces of leukocytes and concentrated along the borders between ECs (1, 2). PECAM is composed of six extracellular immunoglobulin domains (2). A homophilic interaction (i.e., an interaction between neutrophil or monocyte PECAM and endothelial PECAM) is required for TEM. When antibody to PECAM is applied to either the leukocyte or endothelium, TEM is blocked both in vitro and in vivo (3-6). Less is known about the subsequent migration of leukocytes through the endothelial basal lamina and the interstitial matrix. Using quantitative in vitro assays that dissect different steps in leukocyte transit, we now demonstrate an additional role for PECAM just after the leukocyte has crossed the endothelial junction. This role is mediated by a domain of PECAM distinct from those involved in transendothelial migration and appears to involve heterophilic interactions of leukocyte PECAM with components of the extracellular matrix.

Materials and Methods

Construction of Truncated PECAM-IgG Chimeric Proteins. A full-length PECAM-IgG cDNA was constructed by interrupting PECAM cDNA at the putative membrane insertion site with a NotI site and ligating with a cDNA encoding the hinge and CH2 and CH3 regions of human IgG1 (generously provided by Drs. Mickey Hu and Mark Zukowski of Amgen, Inc. Thousand Oaks, CA), and subsequently subcloned into the expression vector pcDNA1/Neo (Invitrogen, San Diego, CA). PC1K technology was used to produce a series of PECAM constructs differing by the addition of a complete 3' domain. A common 5' primer and a series of 3' primers engineered with HindIII and NotI sites, respectively, were used to amplify DNA encoding only the desired segments of PECAM (2, 7). These were then ligated into pcDNA1/Neo and rejoined with hlgG in a separate step. The sequence of the 5' primer was 5'-TCAG TAGACCACCATGCAG-3'. The shaded area represents the HindIII site. The initiator ATG codon is underlined. The sequences of the 3' primers were:

1. 5'-TAGAATATCTGGCGGCGCTTGATGCGCTTCTT-3'
2. 5'-TAGAATATCTGGCGGCGCTTTCCATGATCATTTCC-3'
3. 5'-TAGAATATCTGGCGGCGCTTGGTCCAGATGTGT-3'
4. 5'-TAGAATATCTGGCGGCGCTTCGATGGTCGTCTGCC-3'
5. 5'-TAGAATATCTGGCGGCGCTAGACTCCACACCTT-3'

The shaded areas mark the NotI restriction site.

1Abbreviations used in this paper: EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; Mo, monocytes; N-CAM, neural cell adhesion molecule; Ng-CAM, neuron-glia cell adhesion molecule; PECAM, platelet/endothelial cell adhesion molecule-1; TEM, transendothelial migration.
Production and Assay of Truncated PECAM-IgG Chimeras. Cos
cells were transiently transfected with each of the PECAM-IgG
constructs of the 3' deletion series as well as full-length PECAM-
IgG. 20 μg of plasmid was added to 10^7 Cos cells in 0.5 ml of
DME medium in a 4 mm cuvette and subjected to electropora-
tion at 960 μF, 250 mV in a gene pulser (Bio-Rad, Richmond,
CA) (3). Transfected cell culture supernates were collected and
subjected to SDS-PAGE under nonreducing conditions. The
4-11% gradient gel was electroblotted onto Immobilon mem-
brane (Millipore Corp., Bedford, MA) and probed with an alka-
line phosphatase-labeled goat anti-human Fc antibody and devel-
oped with NBT/BCIP as the substrate (2).

Monoclonal Antibody Binding ELISA. An ELISA assay was de-
veloped to map the epitopes recognized by a panel of anti-PECAM
mAb. Rabbit anti-mouse IgG was adsorbed to ELISA plates fol-
lowed by blocking in phosphate buffered saline + 0.1% ovalbu-
min. 100 μl of monoclonal antibody supernate or 1 μg of puri-
ified IgG were added to each well in a column and allowed to
bind for 1 h at room temperature. After washing, 100 μl of culture
medium from Cos cells transfected with each truncated PECAM-
IgG construct were added to the plates for 1 h at room tempera-
ture. After extensive washing, binding of the PECAM chimeras
was detected using Alkaline phosphatase-labeled goat anti-human
Fc in the Attophos assay, as described (8, 9). MAb P1.1, P1.2, and
P1.3 were provided by Dr. Peter Newman (Blood Center of SE
Wisconsin); mAb 4G6 by Dr. Steven Albelda (University of
Pennsylvania, Philadelphia, PA); mAb L133.1 was purchased
from Becton-Dickinson (San Jose, CA).

Human Umbilical Vein Endothelial Cell Culture, Leukocyte Iso-
lation, and Transendothelial Migration Assays. These were all per-
formed as described in detail in references 1, 10, and 3, respec-
tively. Briefly, human umbilical vein endothelial cells (HUVEC)
were grown to confluence on hydrated Type I collagen gels in
the presence of 20% normal human serum without additional
growth factors. Peripheral blood mononuclear cells were added
to the medium above these monolayers and monocyte-selective
transendothelial migration (10) was allowed to proceed for 1 or 2
hours in the presence of anti-PECAM reagents or controls. The
monolayers were then treated as described (3). Monocytes ar-
ested on the apical surface were identified by their ability to bind

![Figure 1](image-url)

**Figure 1.** (a) Schematic diagram of the strategy for
constructing truncated PECAM molecules. A portion
of the pcDNA1/Neo vector containing cDNA for the
signal sequence (SS, shaded box) and the six extracellu-
lar immunoglobulin domains of PECAM fused at an
engineered Nod site to a sequence encoding the hinge,
C2, and C3 domains of the human IgG heavy chain
(hIgG) is shown. (b) Western blot of series of sequen-
tially truncated PECAM-IgG chimeras. Lanes 1-5 in-
dicate the position of sequentially longer constructs
made by primers 1-5 in part A. Lane 6 shows the full-
length PECAM-IgG. Lane 0 shows supernate from
mock transfected Cos. Numbers on the left indicate the
positions of molecular mass standards. Bands 1-6 run at
the expected positions for dimers of ~80, 110, 140,
170, 200, and 230 kD, respectively. Note that essen-
tially all of the detectable molecules are running as in-
tact dimers. (c) Schematic diagram of PECAM-IgG
constructs 1-6 and the reactivity of various anti-
PECAM mAb, as determined by ELISA assay. Lack of
reactivity above background is indicated by −, whereas all wells with a + were generally 40-100X above background levels. Rabbit anti–mouse mAb
adsorbed to ELISA plates was used to bind the various mAb placed in each well in the vertical column. Cos supernates containing constructs 1-6 or
mock transfected Cos supernate (0) were placed in each row across. Binding of the PECAM chimeras was detected using Alkaline phosphatase-labeled
goat anti–human Fc.

1338 Separate Domains of PECAM Mediate Distinct Phases of Monocyte Emigration
Only mAb binding to PECAM domains 1 and/or 2 block TEM of Mo. PBMC were incubated with the indicated mAb at 20 µg/ml, or heparin or dextran sulfate (Sigma Chemical Co., St. Louis, MO) at 50 µg/ml and added to HEC monolayers for 2 h at 37°C (concentrations previously found to block optimally [3, 14]). The monolayers were washed with EGTA to remove unbound and integrin-bound leukocytes. The percent of cells remaining associated with the HEC that had transmigrated below the monolayer was quantitated as described (3). Approximately 100 cells were counted for each replicate monolayer. The bars represent the mean and standard error for six replicate monolayers. In cases where two mAb were added, the concentration of each was 20 µg/ml. W6/32 is a mAb against Class I MHC antigen used as a negative control (3). PECAM-IgG is the full-length PECAM-IgG chimera containing domains 1-6. Two independent experiments are shown as a and b.

IgG- or C3bi-coated sheep erythrocytes. Transmigrated monocytes were at a lower focal plane and did not bind opsonized erythrocytes (3).

**Interstitial Migration Assay.** To quantitate migration into the gel, the fixed, Wright/Giemsa stained monolayers were examined by Nomarski optics at 400X. The microscope was focused on the apical surface of the monolayer. The fine focus was then moved slowly through serial increments of 5 µm and the number of monocytes (Mo) within that level were scored.

**Electron Microscopy.** Electron microscopy was performed by standard techniques essentially as described (1). Monolayers of HUVEC on collagen were fixed in half-strength Karnovsky’s fixative and postfixed with osmium tetroxide prior to embedding in Epon. Cross-sections of the monolayer were cut on a Reichert-Jung ultramicrotome and viewed on an electron microscope (JEOL 100 CX-II, JEOL U.S.A. Inc., Peabody, MA).

**Results and Discussion**

Anti-PECAM monoclonal antibody (mAb) hec7 blocks TEM of Mo and neutrophils (PMN) in a quantitative in vitro assay (3). The block is selective for TEM. There is no diminution of the ability of the leukocytes to adhere to the apical surface of the ECs; rather, the leukocytes remain tightly adherent over the intercellular borders, reversibly arrested in their transendothelial passage (3). Not all anti-PECAM mAb block TEM; functional studies in conjunction with mAb mapping were used to determine which extracellular domains of PECAM are critical for TEM.

The extracellular portion of PECAM is organized into six Ig domains (2). A series of sequentially truncated PECAM-IgG chimeras were synthesized in order to map the epitopes of a panel of mAb (Fig. 1 a). The truncated PECAM-IgG chimeras ran at the expected molecular mass on nonreduced SDS-PAGE (Fig. 1 b). In an ELISA the mAb P1.3 bound to all of the constructs containing domain 1, including the shortest containing only domain 1 (Fig. 1 c). Binding of hec7 and L133.1 required expression of both domains 1 and 2. P1.1 bound only to the constructs containing domain 5, whereas the mAbs P1.2 and 4G6 bound only to full-length constructs containing domain 6 (Fig. 1 c). We will refer to hec7 and L133.1 as domain 1 and/or 2 mAb and P1.2 and 4G6 as domain 6 mAb, although these constructs alone cannot assign the domain of the epitope unambiguously. Nevertheless, our results are consistent with a recently published study in which a different technique was used to identify the epitopes of anti-PECAM mAb (11).

All mAb used in these experiments bind to Mo equivalently. However, only the three mAb that bound to PECAM domain 1 and/or 2 blocked TEM in our in vitro assay (Fig. 2). In a representative experiment hec7, L133.1, and P1.3 blocked TEM of Mo by 73, 67, and 70%, respectively, whereas mAb P1.1, P1.2, and 4G6 had no effect (Fig. 2). Under the conditions used in these experiments, the mAb exerts its effect by binding to monocyte PECAM rather than the endothelial PECAM (3).

Our assay employs direct visualization of the interacting cells to assess the final positions of Mo in relation to the endothelial monolayer. Mo treated with anti-PECAM mAb that recognized domain 6 migrated across the endothelial monolayer. Once under the monolayer, however, they remained close to the abluminal surface of the endothelium instead of migrating deep into the collagen gels, as Mo treated with control mAb. Fig. 3 quantitates the migration of Mo below the monolayer (interstitial migration). By two hours, control Mo were distributed among all six levels with less than 20% remaining in the uppermost 10 µm. MAb hec7, L133.1, and P1.3 blocked TEM; however, the fraction of Mo that did transmigrate moved into the gel as deeply as control cells. In contrast, 80-90% of the Mo treated with anti-domain 6 mAbs P1.2 or 4G6 were still in the uppermost 10 µm, close to the basal surface of the ECs, after 2 h. Similar results were seen when the incubation time was extended to 4 h (not shown).
These results suggest that TEM and interstitial migration are mediated by separate domains of the PECAM molecule. Combining mAb P1.2 and hec7 did not change the ability of hec7 to block TEM (Fig. 2 b), nor did it alter the ability of P1.2 to block migration of Mo into the collagen gel (Fig. 3 b). The domains required for TEM and interstitial migration are spatially separated, since mAb P1.1, which binds domain 5, had no effect on either function (Figs. 2 and 3).

An experiment identical to that of Fig. 3 was performed, but this time cultures were fixed and embedded in Epon. Depth of Mo migration was quantitated in 1-μm sections cut perpendicular to the endothelial monolayer, and similar results were obtained (not shown). This material was then examined by electron microscopy. Many of the cells treated with anti-domain 6 mAb were retained between the basal surface of the EC and the endothelial basal lamina (Fig. 4 b and c). In

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**Figure 4.** Monoclonal antibodies against different domains of PECAM block monocyte migration at two distinct steps. Mo were allowed to interact with HEC monolayers for two hours in the presence of anti-PECAM mAb hec7 (a), P1.2 (b), or 4G6 (c) all at 20 μg/ml final concentration. Monolayers were then fixed and processed for electron microscopy. a shows the typical profile of an adherent monocyte (Mo) blocked from transmigrating the endothelial cell (EC) junction by mAb hec7, as had been previously demonstrated (3). Small arrows mark the edges of the interendothelial cell border. Large arrows point to the amorphous fuzzy endothelial cell basal lamina (BL). Note monocyte filopodia probing the apical surface of the EC. b and c show the appearance of typical monocytes still in contact with the undersurface of the EC in the presence of anti-domain 6 mAb P1.2 and 4G6, respectively. They have migrated through the interendothelial junction (small arrows) and lie between the basal surface of the EC and the endothelial basal lamina (BL, large arrows). Note filopodia probing through the BL into the underlying collagen gel, which extends well beyond the field of this photograph. CF (open arrow) shows a collagen fibril. Bar, 1 μm.
contrast, mAb hec7 blocked TEM at the apical surface of the EC (Fig. 4 a), as previously demonstrated (3).

As previously mentioned, PECAM on a leukocyte can bind in a homophilic manner to PECAM on the EC (3), but PECAM-transfected L cells can also aggregate in a PECAM-dependent heterophilic manner (12-15). Heparan sulfate proteoglycans are ligands for this heterophilic adhesion. However, to date there has been no known physiologic function for the binding of PECAM to proteoglycan. It was apparent that the ability of mAb to inhibit interstitial migration exactly paralleled their ability to inhibit heterophilic aggregation of PECAM-transfected L cells. In those published experiments, P1.2 and 4G6 inhibited, but hec7, L133.1, P1.1 and P1.3 were inactive (15) (and unpublished results). Heparin at 50 µg/ml (and heparan sulfate, not shown), which blocked heterophilic aggregation (14, 15) similarly inhibited migration through extracellular matrix (Fig. 3). This was a specific effect of heparin, since dextran sulfate, a similarly charged polymer used at the same concentration, had no effect. Neither polymer had a significant effect on TEM (Fig. 2). The site(s) on PECAM that interact with heparin have not been defined. Conversely, full-length PECAM-IgG, which would be expected to interact homophilically with PECAM on Mo blocked TEM (Fig. 2 a) but had no effect on interstitial migration (Fig. 3 a).

We conclude that while the homophilic interaction of the amino-terminal region of leukocyte PECAM with PECAM of the EC is involved in transendothelial migration per se, heterophilic interaction of the membrane-proximal region of leukocyte PECAM with heparan sulfate proteoglycans (such as perlecan) in the endothelial basal lamina, and possibly deeper in the interstitial tissues, is involved in the movement of transmigrated leukocytes through the extracellular matrix. A schematic diagram of the PECAM molecule with its mAb epitopes and putative functional domains is shown in Fig. 5. Whether interaction with proteoglycans serves this function by promoting adhesion directly, via activating leukocyte integrins (9, 16, 17), or by stimulating monocyte secretion of matrix-degrading proteases is under investigation.

Only two other adhesion molecules, neuron-glial cell adhesion molecule (Ng-CAM) (18) and neural cell adhesion molecule (N-CAM) exhibit both homophilic and heterophilic binding functions. N-CAM binds to itself via domain 3 (19) and to heparin via domain 2 (20). The domains responsible for the adhesive interactions of Ng-CAM with itself (18) or the heterophilic ligands laminin (21) and chondroitin sulfate proteoglycans (22, 23) have not been identified. The leukocyte integrin VLA-4 (α4β1) has been shown to bind heterophilically to two different ligands, the endothelial cell adhesion molecule VCAM-1 and the matrix molecule fibronectin via two separate domains (24). The endothelial adhesion molecule MAdCAM-1 supports rolling interactions with lymphocyte α4β7 (25) as well as L-selectin (26), presumably via Ig-like and mucin-like domains, respectively. However, the present report is the first description of a leukocyte-endothelial cell adhesion molecule that utilizes distinct domains for two different mechanisms of interaction (homophilic and heterophilic), to mediate two separate and sequential functions, transendothelial migration and migration through extracellular matrix, respectively.
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