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Antibodies Against the First Ig-Like Domain of Human Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) That Inhibit PECAM-1-Dependent Homophilic Adhesion Block In Vivo Neutrophil Recruitment

Marian T. Nakada,* Kunjlata Amin,† Melpo Christofidou-Solomidou,‡ Christopher D. O’Brien,† Jing Sun,§ Indira Gurubhagavatula,¶ George A. Heavner,* Alexander H. Taylor,** Cathy Paddock,§ Qi-Hong Sun,§ James L. Zehnder,¶ Peter J. Newman,§ Steven M. Albelda,† and Horace M. DeLisser‡

Platelet endothelial cell adhesion molecule (PECAM-1), a member of the Ig superfamily, is found on endothelial cells and neutrophils and has been shown to be involved in the migration of leukocytes across the endothelium. Adhesion is mediated, at least in part, through binding interactions involving its first N-terminal Ig-like domain, but it is still unclear which sequences in this domain are required for in vivo function. Therefore, to identify functionally important regions of the first Ig-like domain of PECAM-1 that are required for the participation of PECAM-1 in in vivo neutrophil recruitment, a panel of mAbs against this region of PECAM-1 was generated and characterized in in vitro adhesion assays and in an in vivo model of cutaneous inflammation. It was observed that mAbs that disrupted PECAM-1-dependent homophilic adhesion in an L cell aggregation assay also blocked TNF-α-induced intradermal accumulation of neutrophils in a transmigration model using human skin transplanted onto SCID mice. Localization of the epitopes of these Abs indicated that these function-blocking Abs mapped to specific regions on either face of domain 1. This suggests that these regions of the first Ig-like domain may contain or be close to binding sites involved in PECAM-1-dependent homophilic adhesion, and thus may represent potential targets for the development of anti-inflammatory reagents. The Journal of Immunology, 2000, 164: 452–462.

The recruitment of neutrophils out of the circulation into sites of acute inflammation is considered to involve a complex set of sequential events mediated by soluble inflammatory mediators and specific groups of cell adhesion proteins (1–4). The process begins with the leukocyte rolling along the surface of the endothelium, a step that has been previously described as mediated by the selectins and their carbohydrate-rich counterligands. As the leukocyte rolls, L-selectin is shed and bind to seven-transmembrane-domain, G protein-coupled receptors on the leukocyte (5). Leukocyte activation leads to arrest of rolling and firm adhesion of the leukocyte to the endothelium, events that result from the binding of integrins expressed on the leukocytes to Ig superfamily members expressed on the endothelium. Once the leukocyte has adhered to the endothelium, it migrates over the luminal surface, locates an endothelial intercellular junction, and then squeezes between endothelial cells and through the matrix of the basement membrane to enter the extravascular space.

A number of leukocyte and endothelial cell adhesion molecules contribute to neutrophil emigration, including PECAM-1 (CD31) (reviewed in Refs. 6 and 7). PECAM-1, a transmembrane glycoprotein of the Ig superfamily, is expressed on the surface of leukocytes and platelets and on the endothelium, where it concentrates at cell-cell borders. Its extracellular region is composed of six Ig-like domains, while its long cytoplasmic tail has several serine and tyrosine residues whose phosphorylation may be important in regulating the activity of the molecule. PECAM-1 functions as both an adhesion molecule and a signal transducer through binding interactions with itself (homophilic adhesion) or with other non-PECAM-1 molecules (heterophilic adhesion) (6, 7).

The involvement of PECAM-1 in in vivo inflammation (reviewed in Ref. 8) has been established in a number of studies that have demonstrated that soluble forms of PECAM-1 or anti-PECAM-1 Abs block neutrophil recruitment in several animal models of acute inflammation (9–15). The mechanism of the involvement of PECAM-1, however, is currently still being defined. In all of the studies noted above, neutrophils were observed to accumulate within the vessels and/or in subendothelial areas, but were strikingly absent from extravascular sites, suggesting that in

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1 Abbreviations used in this paper: PECAM-1, platelet endothelial cell adhesion molecule-1; huPECAM-1, human PECAM-1; 125I-anti-mouse, 125I-labeled anti-mouse; MFI, mean fluorescence intensity; muPECAM-1, murine PECAM-1.
vivo, PECAM-1 is involved in the migration of neutrophils across the endothelium and perivascular basement membrane. Leukocyte transendothelial migration involves two steps: initial passage through endothelial intercellular junctions (diapedesis), followed by movement across the basement membrane. PECAM-1 may be required for both steps of this process, as Liao et al. (16) have shown in in vitro transendothelial migration studies that mAbs that blocked PECAM-1 homophilic adhesion inhibited movement across the endothelium, while Abs that inhibited heparin-inhibitable PECAM-1 heterophilic adhesion blocked passage through extracellular matrix.

We and others have shown that the first Ig-like domain of PECAM-1 is required for homophilic adhesion (17–19). A number of residues in domain 1 have been implicated in homophilic adhesion (19); however, their importance in mediating PECAM-1 function during in vivo inflammation is not known. Therefore, to define functionally important regions in domain 1, a panel of murine mAbs against the first Ig-like domain of huPECAM-1 was generated and characterized in terms of their effects in in vitro adhesion studies as well as on in vivo neutrophil accumulation in a model of human skin transplanted onto SCID mice. It was noted that Abs that disrupted homophilic adhesion also blocked TNF-α-induced intradermal accumulation of neutrophils. The epitopes of these functionally active Abs mapped to specific regions on either face of the first Ig-like domain. These regions may represent potential targets that could be exploited for the development of anti-inflammatory PECAM-1 antagonists.

Materials and Methods

Generation of murine mAbs against the first Ig-like domain of huPECAM-1

A huPECAM-1 cDNA fragment encoding Ig-like domains 1 and 2 was ligated into cDNA encoding the human IgG4 C region using PCR amplification methodology. The resulting construct was expressed in SP2/0 murine hybridoma cells, and recombinant protein (designated 1,2PECAM-1-IgG) was purified to homogeneity from culture supernatants by protein A chromatography. Mice were immunized and boosted with the 1,2PECAM-1-IgG fusion protein and spleen cells were isolated and fused with murine hybridoma cells, and recombinant protein (designated 1,2PECAM-1-IgG) was purified to homogeneity from culture supernatants by protein A chromatography. Mice were immunized and boosted with the 1,2PECAM-1-IgG fusion protein and spleen cells were isolated and fused with murine hybridoma F0 cells (American Type Culture Collection, Manassas, VA). Clones producing anti-PECAM-1 mAbs were selected by ELISA for their reactivity with immobilized recombinant 1–2PECAM-1-IgG4 and their lack of reactivity with control IgG4 fusion protein. These clones were subcloned, and single clones were isolated with subsequent purification of the secreted mAb.

Preparation of Fab fragments by papain digest

Whole Ig was digested in PBS with 10 mM cysteine and 5 mM EDTA at pH 8.8. Papain (Boehringer Mannheim, Indianapolis, IN) was activated by dilution to 1 mg/ml in PBS containing 10 mM cysteine and 5 mM EDTA at pH 8.8 and incubating in a 37°C water bath for 30 min. Activated papain was added to Ig at 1% (w/w), and the digest mixture was maintained at 37°C. The digest was monitored by SDS-PAGE and quenched with 20 mM iodoacetamide upon completion. Fab was purified from Fc and fragments by protein A affinity chromatography.

Construction of human/mouse PECAM-1 chimeric constructs

A total of six human/mouse chimeric mutants are described in this work. The constructs are named by assuming a huPECAM-1 backbone followed by a listing of any regions of the molecule that contain mouse sequences. Thus, huPECAM-1(D1/1–25mu) represents huPECAM-1 construct in which Ig-like domain 1 has been replaced by muPECAM-1, while huPECAM-1(D1/1–25mu) designates a huPECAM-1 mutant in which aa 1–25 of Ig-like domain 1 have been replaced by the homologous muPECAM-1 sequence.

The preparation of these constructs has been previously described (17).

huPECAM-1(D1/1–25mu), huPECAM-1(D1/1–45mu), huPECAM-1(D1/46–103mu), and huPECAM-1(D1/71–103mu)

To generate mutant cDNAs of huPECAM-1 in which sequences of domain 1 were replaced by the corresponding sequences from domain 1 of muPECAM-1 (see Fig. 4), the cDNA of huPECAM-1 in the PBK.CMV expression vector (Stratagene, La Jolla, CA) was cut with two unique restriction endonucleases, ApaI and BstEII. ApaI cuts early in the signal sequence at bp 159, while BstEII cuts at bp 819, a site between the second and third Ig-like domains. Replacement sequences were then engineered for each construct in a two-step process in which two PCR-generated sequences (5’ fragment and 3’ fragment) were joined together by means of sequence overlap extension (20). The gel-purified 5’ and 3’ fragments were then used as templates and joined together to create inserts using the forward primer for the 5’ fragment and the reverse primer for the 3’ fragment. The replacement sequences were then digested with ApaI and BstEII and ligated into the previously digested huPECAM-1/PBK.CMV construct. (The primers and templates used to generate these constructs are available upon request.) All of the mutant constructs were sequenced in the regions of change, and protein expression was confirmed in COS cells by immunofluorescence staining. A mesothelioma line, REN, was subsequently transfected with these constructs using previously described procedures (21). Protein expression was confirmed by FACS analysis and immunoprecipitation. Unless otherwise stated, each clone studied expressed PECAM-1 on its surface on greater than 90% of the cells.

Aggregation of L cell transfectants

The aggregation assay used in these studies has been described in detail previously (17). Briefly, stable L cell transfectants, which had been plated (8–10 10 cells/75-cm2 flask) and grown overnight, were removed with trypsin for huPECAM-1 constructs and nonenzymatically for muPECAM-1 constructs. The cells were washed twice with 10 mM EDTA in PBS, pH 7.2, and twice with HBSS without divalent cations. Cells were finally resuspended to a concentration of ~0.8–1 106/ml in HBSS with or without 1 mM calcium. After the cells had been dispersed to a single cell suspension, 1-ml aliquots with and without Ab (concentration 100 μg/ml) were transferred to wells in a 24-well nontissue culture plastic tray (Costar, Cambridge, MA) that had been previously incubated with 2% BSA in HBSS for at least 1 h and washed thoroughly with HBSS immediately before use to prevent nonspecific binding to the plastic of the tray. The trays containing the suspended L cells were rotated on a gyroratory platform (100 rpm) at 37°C for 30 min. Aggregation was quantified by examining representative aliquots from each sample on a hemocytometer grid using phase-contrast optics. The number of single cells (cells in aggregates 23) remaining vs those present in aggregates of greater than three cells were counted from four 1-mm squares. At least 400 cells were counted from each sample. The percentage of total cells present in aggregates was used as the measure of aggregation. For comparison, the aggregation data for each mAb were subsequently expressed as the percentage of control aggregation, which was calculated by dividing the aggregation in the presence of the mAb by the mean control aggregation in the absence of mAb.

Binding of PECAM-1/IgG chimera to cellular transfectants

The generation of the PECAM-1/IgG chimera has been previously described (18). A total of 5 × 106 cells in 100 μl of HBSS containing 10% FBS with or without Ab (100 μg/ml) was mixed with 5 μg of PECAM-1/IgG in 25 μl of HBSS and allowed to incubate for 90 min at 22°C. Cells were then washed by centrifugation with 1 ml of HBSS and then resuspended in 100 μl HBSS containing 0.8 ml of FITC-labeled goat anti-human IgG chain (Jackson ImmunoResearch, West Grove, PA). Following a 30-min incubation at 4°C, the cells were washed once more in HBSS, resuspended in 0.6 ml of ice-cold HBSS, and subjected to flow-cytometric analysis using FACSscan (Becton Dickinson, San Jose, CA) (18). For Ab studies, Ab concentration was 10 μg/ml.

FACS analysis

The various cell lines used in this study were treated with various anti-human or anti-murine PECAM-1 mAbs for 1 h at 4°C. The primary Ab was then removed, the cells were washed with PBS, and a 1/200 dilution of FITC-labeled goat anti-mouse or anti-rat secondary Ab (ICN Pharmaceutical, Aurora, OH) was added for 30 min at 4°C. After washing in PBS, flow cytometry was performed using an Ortho Cytofluorograph 50H cell sorter equipped with a 2150 data handling system (Ortho Instruments, Westwood, MA).
Skin transplantation

The protocols for skin transplantation have been described previously in detail (13, 22). Briefly, SCID mice were obtained from a colony maintained at the Wistar Institute Animal Facility. At 4–6 wk of age, the plasma of each mouse was tested for IgM production and only fully immunodeficient mice were used. Neonatal foreskins from elective circumcisions obtained using sterile techniques were cut into two halves and trimmed to a diameter of 1–1.5 cm. Full-thickness human skin grafts from the same donor were then transplanted into full-thickness, size-matched wound beds prepared on each flank of a SCID mouse. Mice were used for experiments only within the second month following human skin transplantation to ensure engraftment and stabilization. Only those mice whose grafts grossly showed no signs of inflammation or rejection were used.

Immunoperoxidase staining

Immunoperoxidase staining was performed as previously described (13, 22). Counterstaining was not used to enhance the detection of positive cells.

Quantification of leukocyte infiltration

To identify murine leukocytes, frozen sections of skin grafts were stained as described above with the anti-mouse Mac-1 mAb, MI/70.15, which does not cross-react with human Mac-1 (13). Four to six sections were cut from the center of each skin biopsy, as marked by colloidal carbon (see below), to obtain representative samples of the injection site. The number of leukocytes per 3–6 microscope fields (×200) within close proximity of the colloidal carbon in each section were directly counted to give an estimate of the number of leukocytes. Because some grafts demonstrated a cuff of leukocytes at the murine-human skin junctional regions, the areas counted were in the immediate subepidermal regions not adjacent to murine skin.

Effect of Abs on TNF-induced migration of murine leukocytes

The protocols for this model of TNF-induced neutrophil recruitment have been described previously (13, 22). A skin graft on one side of each mouse was injected intradermally with 50 μl of endotoxin-free saline containing 2 mg/ml of BSA (Sigma, St. Louis, MO) as a protein carrier plus colloidal carbon (Sigma) to mark the site of injection (control side). The skin graft on the contralateral side was injected with 6000 U of human TNF-α (Boehringer Mannheim), diluted in 50 μl of endotoxin-free saline containing 2 mg/ml BSA with colloidal carbon (experimental side). Abs, as Fab fragments, were injected i.v. via tail vein in a final volume of 100 μl with endotoxin-free saline containing 2 mg/ml BSA immediately before the intradermal skin injections. Mice were sacrificed 6 h after saline or TNF-α intradermal injection, and the skin grafts were carefully dissected from the animals. The grafts were cut into two halves through the center of the injection site marked by the colloidal carbon. Each half was oriented, placed in OCT compound, and snap frozen for immunohistochemical analysis, as described above.

mAb biotinylation

Protein A-purified anti-PECAM-1 mAbs were biotinylated by reaction with normal human serum/biotin according to manufacturer’s specification (Boehringer Mannheim). Following dialysis, biotinylated anti-PECAM-1 mAbs were tested for their ability to bind ELISA wells that had previously been coated with 5 μg/ml of 1,2PECAM-IgG4 in bicarbonate buffer. The assays were developed by incubation first with avidin-HRP and then the chromogenic substrate o-phenylenediamine (Sigma). For each biotinylated mAb, the concentration corresponding to the upper linear portion of the titration curve was determined and was selected as the labeled mAb concentration used in subsequent competitive binding assays. In most cases, this standard concentration was about 1 μg/ml.

Competitive Ab-binding studies

Anti-PECAM-1 mAbs with related epitope specificity were identified by pairwise competitive binding studies, as follows. ELISA wells were coated with 5 μg/ml of 1,2PECAM-IgG4 in bicarbonate buffer and blocked with TBS containing 0.05% Tween 20. Each of the unlabeled mAbs was titrated with the standard, nonsaturating amount of the individual biotinylated mAbs determined above. Ab pairs that could reciprocally and completely inhibit binding of each other were considered to recognize overlapping, spatially related epitopes.

Determination of species cross-reactivity

Primary and endothelial cell lines form the indicated species were purchased from Cell Systems (Kirkland, WA) and grown to confluence in 96-well tissue culture plates. Media were removed, and murine anti-PECAM-1 Abs (20 μg/ml) were added and incubated for 1 h at 37°C. Cells were washed twice with media, and 1 μg/ml of radiolabeled 125I-anti-mouse secondary Ab (goat anti-mouse IgG F(ab’2), Jackson ImmunoResearch) with a sp. act. of 1 mCi/ml was added and incubated for 30 min at 37°C. Unbound Ab was removed with three washes, and the radioactivity in each well was counted.

Creation of a molecular model of domains 1 and 2 of huPECAM-1

Molecular modeling was done using Sybyl 6.2 on an SGI Crimson R44000-150 with VGXT graphics. Molecular alignment was done with MegAlign from DNASTar (Madison, WI). The sequence of VCAM-1 was aligned with PECAM-1. Using the crystal structure of the first two domains of VCAM-1 (23) and the sequence alignment, amino acids in VCAM-1 were electronically mutated to the PECAM-1 sequence. Each change was visually examined for steric and electronic interactions. Where unfavorable interactions existed, the side chains of the relevant amino acids were automatically repositioned using Sybyl. There were four regions of PECAM-1 that differed in amino acid number from VCAM-1, two requiring the addition of amino acids and two requiring deletions. For each region, a computer-generated loop search was done using anchoring residues 5 amino acids from either end of the altered regions. The results of each loop search were individually electronically grafted into the PECAM-1 model, and the resulting structures were examined for steric and electronic compatibility. Most structures were eliminated based on backbone-backbone or backbone-side chain interactions. In all instances, the remaining structures were similar. Where severe side chain-side chain interactions were present, side chains were automatically repositioned. The most energetically favored structures were selected and combined in the PECAM-1 model. The crystal structure of VCAM-1 does not contain amino acids corresponding to the first seven amino acids of PECAM-1. To fill in this region, a BLAST search was done using PECAM-1[1–16]. Several similar structures were identified. The sequence 37–52 of Neutral Protease (E.C.3.4.24.27, PDB1NPC.ENT) was selected and grafted on the N terminus of the PECAM-1 model using a least square fit of the α carbons of the sequence NSV (PECAM-1[8–10]) for positioning. Essential hydrogens and charges were added to the structure. The model was subjected to 250 cycles of minimization (steepest descent, NB cutoff 8 Å, dielectric 10). The resulting structure was solvated with water and subjected to an additional 100 cycles of minimization (steepest descent). This was followed by 50 fs of dynamics simulation (300 K, NB cutoff 8 Å, dielectric 10, random velocity), 100 cycles of minimization (steepest descent), and 1000 cycles of conjugate gradient minimization.

Statistical analysis

Differences among groups were analyzed using one-way ANOVA. When statistically significant differences were found (p < 0.05), individual comparisons were made using the Bonferroni/Dunn test.

Results

Generation of murine mAbs against the first Ig-like domain of huPECAM-1

A panel of murine mAbs was generated using standard fusion procedures following immunization with recombinant protein composed of Ig-like domains 1 and 2 of huPECAM-1 fused to an Fc region of human IgGy4 isotype (1,2PECAM-1-IgG). Using cellular transfectants expressing mutant forms of huPECAM-1, six of these Abs (mAbs 24, 35, 37, 40, 51, and 62) were found to bind only to the Ig-like domain 1 (Fig. 1) and were subsequently studied in more detail. All Abs were of the IgG1 isotype except for mAb 62, which was IgG2a.

Domain I anti-PECAM-1 Abs have different effects on PECAM-1-dependent adhesion

The Abs were first investigated for their effects on PECAM-1-dependent adhesion. Initial adhesion studies were done with an aggregation assay involving L cell fibroblasts transfected with PECAM-1 (17, 24). Previous studies with this system have demonstrated that L cell transfectants expressing the full-length
human skin (neonatal foreskin) is transplanted onto SCID mice. A model of the human vasculature has been developed in which accumulation homophilic L cell aggregation also inhibit in vivo neutrophil recruitment in response to intradermal TNF-α. Domain 1 anti-PECAM-1 Abs that inhibit PECAM-1-dependent L cell aggregation while mAbs 37 and 51 did not, these data suggest the ability to disrupt PECAM-1-mediated homophilic adhesion as defined by the L cell assay may correlate with the ability of huPECAM-1 to mediate cell-cell aggregation that involves, on opposing cells, the interaction of huPECAM-1 with an unidentified non-PECAM-1 ligand (possibly a heparin-containing proteoglycan) (heterophilic adhesion). In contrast, a mutant missing exon 14 (huPECAM-1Δ14) exclusively forms cellular aggregates that are the result of PECAM-1–PECAM-1-binding interactions (homophilic adhesion). Given this, the Abs were therefore tested for their effect on PECAM-1-dependent L cell aggregation mediated by either huPECAM-1 or huPECAM-1Δ14 (Fig. 2). Five of the six Abs (100 µg/ml) inhibited PECAM-1-dependent L cell aggregation: three of these Abs, mAbs 24, 40, and 62, blocked L cell aggregation triggered by huPECAM-1 and huPECAM-1Δ14 (heterophilic and homophilic aggregation), while the other two Abs, mAbs 37 and 51, only inhibited aggregation mediated by huPECAM-1 (heterophilic aggregation). Unlike the other Abs, mAb 35 increased the aggregation seen with transfectants expressing either of the PECAM-1 constructs.

The Abs (as Fab fragments) were studied further in a second assay that involved the adhesion of a chimeric protein composed of the extracellular domain of huPECAM-1 fused with human IgG Fc (PECAM/IgG) to huPECAM-1 transfectants (18) (Fig. 3). All of the Abs blocked the binding of the PECAM/IgG chimera except for mAb 35, which appeared to augment adhesion (by 145.5 ± 6.5% compared with control; n = 2). (The effects of the Abs in these two adhesion assays are summarized in Fig. 4.)

In another aggregation assay, L1/2 mouse lymphoma cells transfected with huPECAM-1, but not control cells, spontaneously form large aggregates in the presence of serum or laminin (25). In contrast to the systems described above, mAb 51, but none of the other five Abs, inhibited the aggregation L1/2 mouse lymphoma cells transfected with huPECAM-1 (data not shown).

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FIGURE 1. Ab binding to cellular transfectants expressing various forms of PECAM-1. The domain 1 mAbs were tested for their ability to bind to cellular transfectants expressing huPECAM-1, mutPECAM-1, huPECAM-1(D1mu), or huPECAM-1(D2mu) by FACS analysis. Shown in parentheses for each construct is the ratio of the MFI of Ab binding to the MFI of background staining. +, Indicates Ab binding; −, indicates no Ab binding. For Abs that bound to a given transfectant, the MFI was at least 6-fold greater than that of background. mAbs 2H8 and 390 are two mAbs against mutPECAM-1 with epitopes respectively in domain 1 and domain 2 (H. DeLisser and S. Albelda, unpublished observations). mAb 4G6 is an Ab whose binding epitope is known to be in domain 6 of huPECAM-1 (35). Six of newly generated Abs (24, 35, 37, 40, 51, 62) bound only to constructs that contained the human sequences for Ig-like domain 1. Each FACS analysis was repeated at least once with virtually identical results.

huPECAM-1 mediates cell-cell aggregation that involves, on opposing cells, the interaction of huPECAM-1 with an unidentified non-PECAM-1 ligand (possibly a heparin-containing proteoglycan) (heterophilic adhesion). In contrast, a mutant missing exon 14 (huPECAM-1Δ14) exclusively forms cellular aggregates that are the result of PECAM-1–PECAM-1-binding interactions (homophilic adhesion). Given this, the Abs were therefore tested for their effect on PECAM-1-dependent L cell aggregation mediated by either huPECAM-1 or huPECAM-1Δ14 (Fig. 2). Five of the six Abs (100 µg/ml) inhibited PECAM-1-dependent L cell aggregation: three of these Abs, mAbs 24, 40, and 62, blocked L cell aggregation triggered by huPECAM-1 and huPECAM-1Δ14 (heterophilic and homophilic aggregation), while the other two Abs, mAbs 37 and 51, only inhibited aggregation mediated by huPECAM-1 (heterophilic aggregation). Unlike the other Abs, mAb 35 increased the aggregation seen with transfectants expressing either of the PECAM-1 constructs.

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In another aggregation assay, L1/2 mouse lymphoma cells transfected with huPECAM-1, but not control cells, spontaneously form large aggregates in the presence of serum or laminin (25). In contrast to the systems described above, mAb 51, but none of the other five Abs, inhibited the aggregation L1/2 mouse lymphoma cells transfected with huPECAM-1 (data not shown).

Domain 1 anti-PECAM-1 Abs that inhibit PECAM-1-dependent homophilic L cell aggregation also inhibit in vivo neutrophil accumulation

A model of the human vasculature has been developed in which human skin (neonatal foreskin) is transplanted onto SCID mice (13, 22). The vessels of the grafts remain principally human for several months, while the circulating murine leukocytes (principally neutrophils) retain the ability to interact with the human vasculature and thus are able to participate in an inflammatory reaction. In this model, two halves of the same foreskin are transplanted onto the back of each SCID mouse. Intradermal injection of TNF-α induces a 2–4-fold increase in the number of leukocytes accumulating in the dermis compared with grafts injected with saline. Using this model, we have demonstrated that Ab against huPECAM-1 blocks TNF-α-induced recruitment of neutrophils into the skin grafts (9, 13). To our knowledge, this model represents the only system available to look at the response of the human vasculature to an inflammatory stimulus in an in vivo context, although this assay is limited by the requirement that the human molecules must be able to interact with the homologous mouse counter-receptor.

Therefore, to evaluate the effect of these Abs on in vivo inflammation, we studied their effect on the accumulation of neutrophils in TNF-α-stimulated skin grafts (Fig. 5). As these Abs do not recognize muPECAM-1 (Fig. 1), in human/SCID mice chimeras they bind only to huPECAM-1 on the human vasculature of the graft. As previously observed in animals given BSA i.v., the intradermal injection of TNF-α, but not saline, resulted in significant accumulation of leukocytes within the dermis (9, 13). However, i.v. injection of mAb 24, 40, 35, and 62 Fab fragments (100 µg Ab/mouse) significantly blocked the TNF-α-induced extravasation of leukocytes into the dermis (p < 0.05). In contrast, mAb 37 and 51 Fab fragments (100 µg Ab/mouse) did not significantly impair neutrophil recruitment in response to intradermal TNF-α. Fab fragments were used for these experiments, and thus the effects of the blocking Abs were unlikely to be due to Fc interactions. The Abs, as Fab fragments, did not induce neutropenia. As mAbs 24, 40, 35, and 62 blocked or augmented PECAM-1-dependent homophilic L cell aggregation while mAbs 37 and 51 did not, these data suggest that the ability to disrupt PECAM-1-mediated homophilic adhesion as defined by the L cell assay may correlate with the ability of...
the domain 1 Abs to block in vivo inflammation in the human skin/SCID mouse model.

Localization of the binding epitopes of the domain 1 anti-PECAM-1 Abs
To better understand the basis of the differential effects of these domain 1 Abs, their binding epitopes were localized. To accomplish this, we employed a sequential strategy that involved: 1) competitive Ab inhibition; 2) analysis of Ab binding to human/mouse PECAM-1 chimeric mutants; and 3) Ab species cross-reactivity and comparisons of the amino acid sequences of human, rat, bovine, and PECAM-1.

Four pairs of domain 1 anti-PECAM-1 Abs are identified by competitive Ab-binding studies
We first studied the ability of unlabeled Abs to compete for the binding of biotinylated Abs to 1,2-PECAM-1-IgG recombinant protein. From these studies, four pairs of cross-blocking Abs (24 and 40; 37 and 51; 35 and 62; and 51 and 62) were defined (Fig. 6). These data suggested that the Abs in each pair bound to the same epitope or to epitopes that were spatially close to each other.

FIGURE 2. Effect of domain 1 anti-PECAM-1 Abs in the L cell aggregation assay. Domain 1 anti-PECAM-1 Abs were tested for their effect on PECAM-1-dependent L cell aggregation mediated by either huPECAM-1 (heterophilic aggregation) or huPECAM-1Δ14 (homophilic aggregation). Data are presented as the mean percent of control aggregation for each Ab (see Materials and Methods) (n = 2–4). mAb 1.1 had no effect on PECAM-1-dependent L cell aggregation. mAbs 24, 40, and 62 blocked L cell aggregation triggered by huPECAM-1 and huPECAM-1Δ14. mAbs 37 and 51 inhibited only aggregation mediated by huPECAM-1. mAb 35 increased by at least 150% the aggregation seen with transfectants expressing huPECAM-1 or huPECAM-1Δ14. Data are presented as mean ± SEM. Statistical differences were calculated by ANOVA. *, p < 0.05 when compared with control aggregation in the absence of Ab (No Ab).

FIGURE 3. Effect of domain 1 anti-PECAM-1 Abs on the binding of PECAM/IgG to PECAM-1-expressing cellular transfectants. Domain 1 anti-PECAM-1 Abs were tested for their effect on the adhesion of PECAM/IgG to huPECAM-1 transfectants using FACS analysis and FITC-conjugated goat anti-human IgG. The MFI was used as the index of PECAM/IgG binding. Compared with control without Ab, all of the Abs blocked the binding of the PECAM/IgG chimera, except for mAb 35, which appeared to augment adhesion. Data shown are representative of two experiments.

FIGURE 4. Effect of domain 1 anti-PECAM-1 Abs in different assays of PECAM-1-dependent adhesion. The effect of the domain 1 anti-PECAM-1 Abs on PECAM-1-dependent adhesion in two distinct assays (see Materials and Methods) was compared (↓ = inhibition of adhesion; ↑ = augmentation of adhesion; NE = no effect). Five of the six Abs inhibited PECAM-1-dependent L cell aggregation: three (mAbs 24, 40, and 62) blocked both homophilic and heterophilic interactions, while two (mAb 37, 51) inhibited only heterophilic interactions. mAb 35 increased PECAM-1-dependent L cell aggregation. All of the Abs completely blocked the binding of the PECAM/IgG chimera to PECAM-1-expressing transfectants, except for mAb 35, which appeared to augment adhesion.
The binding epitopes for the domain 1 anti-PECAM-1 Abs are clustered in two distinct linear sequences

To locate the linear sequences within domain 1 that contained the binding epitopes, we took advantage of the fact that these Abs do not recognize muPECAM-1 and determined their binding to chimeric receptors made by interchanging homologous regions of domain 1 from mouse and human PECAM-1. This approach is particularly appealing for epitope analysis, as the generation of chimeric human/mouse PECAM-1 receptors results in mutants in which the tertiary structure is presumably preserved. Analysis of the pattern of Ab binding to these chimeric constructs indicated that the binding epitopes for the Abs were located in two distinct but separated sequences (aa 26–45 and aa 71–103) (Fig. 7). Specifically, mAbs 37, 51, and 62 bound to constructs containing the human sequence for aa 26–45, while mAbs 24, 40, and 35 bound to mutants that carried the human sequence for aa 71–103.

Presumptive epitopes identified by sequence comparisons of human, rat, bovine, and mouse PECAM-1 and Ab binding to different species of endothelial cells

The Abs were tested for their ability to bind to endothelial cells derived from human, rat, bovine, and murine sources (Fig. 8). All of the Abs recognized PECAM-1 on human but not murine endothelial cells (see also Table I). mAb 37 also bound rat endothelial cells, while 62 also bound to endothelial cells of rat and bovine sources. The species reactivity of these Abs coupled with the data described above allowed us to map the presumed binding epitopes by comparing the amino sequences for the different species.

Fig. 9A shows the amino acid sequence in domain 1 (aa 26–45) that contains the epitopes for mAbs 37, 51, and 62. As mAb 62 binds to human, rat, and bovine but not murine endothelial cells, it must recognize a sequence that is common to human, rat, and bovine but not murine PECAM-1. The sequence, LTLQC, best fulfills this requirement. mAb 37 binds to endothelial cells derived from human and rat sources, but not to those from bovine or murine sources. It therefore must bind to a sequence that is present in human and rat but not bovine or murine PECAM-1. The sequence most consistent with this is PQHQ. mAb 51 recognizes only huPECAM-1, but its epitope must be spatially close to mAb 37, as the two competitively inhibit the binding of each other. Given this, a review of the sequences suggests that the epitope for mAb 51 includes HVKP.

Fig. 9B shows the amino acid sequence in domain 1 that contains the epitopes for mAbs 24, 35, and 40 (aa 71–103). Comparison of the human and murine sequences indicates that from aa 77–96 there is a high degree of homology, while there are two regions (EVRIYD, aa 71–76 and QLLVE, aa 97–101) in which the two sequences differ significantly. This suggests that the binding epitopes for these three Abs are located in these latter sequences.
FIGURE 7. Localization of the linear epitopes of the domain 1 anti-PECAM-1 Abs. The domain 1 mAbs were tested for their ability to bind to mutants of huPECAM-1, in which sequences of domain 1 were replaced by the homologous sequence in muPECAM-1. A. The sequences mutated were amino acids (AA) 1–26, 1–46, 46–103, and 71–103, and are shown schematically. Ab binding to each construct was initially determined by immunofluorescence staining and then subsequently confirmed by FACS analysis, with the exception of the huPECAM-1(71–103mut) mutant, in which, for technical reasons, the pattern of Ab binding was confirmed only by immunofluorescence staining (indicated by *; data available upon request). The MFI of Ab binding to a given construct was determined, and the data were expressed as the percentage of the MFI for mAb 4G6 control binding, except for muPECAM-1, in which the data is the percentage of the MFI for mAb 390 binding. These data are shown in the parentheses, and for each construct is of a representative experiment for that mutant in which all of the Abs were tested concurrently.

B, Based on the pattern of binding, it was concluded that the epitopes for mAbs 37, 51, and 62 are located between aa 26 and 46, while those of mAbs 24, 35, and 40 are found between aa 71 and 103.

FIGURE 8. Ab binding to different species of endothelial cells. The species cross-reactivity of the mAbs was determined by treating endothelial cells from different species with the various Abs, followed by incubation with $^{125}$I-anti-mouse IgG. The data represent the means of triplicate determinations and are presented as the percentage of control $^{125}$I-anti-mouse IgG binding in the absence of primary Ab. All of the Abs bound to HUVEC. mAb 37 also bound rat EC, while 62 also bound to EC of rat and bovine sources. Data are presented as mean ± SEM. Statistical differences were calculated by ANOVA. *, p < 0.05 when compared with control $^{125}$I-anti-mouse IgG binding in the absence of Ab (No Ab).
Polymorphism of PECAM-1 has been observed in which a frequent CTG→GTG substitution has been found at codon 125, resulting in alleles that code either leucine or valine at aa 92 in wild-type huPECAM-1 (26). We noted that mAbs 24 and 40 bound 1.5 times more strongly to cells that were homozygous for valine than those that were homozygous for leucine at this site, suggesting that the epitopes for 24 and 40 were located at QLLVE. Given that mAb 35 did not competitively inhibit the binding of mAbs 24 and 40, this suggested that epitope for mAb 35 was located in the sequence EVRIYD.

Discussion
To identify functionally important regions of the first Ig-like domain of PECAM-1 that are required for the participation of PECAM-1 in in vivo neutrophil recruitment, a panel of mAbs against this region of PECAM-1 was generated and characterized in vitro adhesion assays and in an in vivo model of cutaneous inflammation. We observed that Abs that disrupted (blocked or augmented) PECAM-1-dependent homophilic adhesion in the L cell aggregation assay also blocked TNF-α-induced intracellular accumulation of neutrophils in a model of human skin transplanted onto SCID mice. Localization of the epitopes of these Abs indicated that these function-blocking Abs mapped to specific regions of domain 1, regions that may contain or be close to the binding sites for PECAM-1-dependent homophilic adhesion, and thus may represent potential targets for the development of antiinflammatory reagents.

The involvement of PECAM-1 in acute inflammation (8) has been established by studies that have shown that inhibition of PECAM-1 with Abs or soluble PECAM-1 constructs blocks neutrophil (and monocyte) recruitment in models of peritonitis (9–11, 13–15), acute lung injury (9), myocardial ischemia-reperfusion injury (11, 12), and cutaneous inflammation (9, 13). Recently, Liao et al. (14) have demonstrated that a chimeric protein composed of the first Ig-like domain PECAM-1 fused to an IgG Fc region, which presumably binds only homophilically, blocks leukocyte recruitment in chemical-induced peritonitis. Our finding that Abs against domain 1 that block homophilic adhesion in the L cell aggregation assay are also active in vivo is therefore consistent with their observations. Interestingly, neutrophil extravasation into extravascular sites was associated with a loss of PECAM-1 from the surface of extravasated leukocytes (13, 27). Given these observations, understanding the mechanism(s) and the ligand-binding interactions that are involved in these processes has been a subject of recent investigation.

Based on its concentration at intercellular junctions, ability to transduce intracellular signals, and its multiple potential ligand-binding interactions, two distinct levels of involvement in leukocyte recruitment have been proposed for PECAM-1. First, PECAM-1/L1 interactions between the neutrophil and the endothelium facilitate movement of the leukocyte across the endothelium. These homophilic interactions could activate integrin-mediated adhesion to the endothelium or make endothelial intercellular junctions permeable to neutrophils by increasing intercellular calcium. Second, following passage through endothelial cell-cell junctions, the binding of neutrophil PECAM-1 to one of its yet to be identified non-PECAM-1 ligands triggers the movement of neutrophils through the basement membrane possibly by stimulating integrin-dependent migration or protease release. Evidence supporting this proposal has come from in vitro studies of neutrophil transendothelial migration that demonstrated that Abs that blocked PECAM-1 homophilic adhesion inhibited movement across the endothelium, while Abs that inhibited heparin-inhibitable PECAM-1 heterophilic adhesion blocked passage through extracellular matrix (16).

A critical element of this model is the presumed ability of PECAM-1 to bind to itself (homophilic adhesion) or other non-PECAM-1 molecules (heterophilic adhesion). Data from a number of assay systems have convincingly established that PECAM-1 is able to bind homophilically (reviewed in Ref. 6). Evidence indicates that PECAM-1-dependent homophilic adhesion involves the direct interaction of the first two extracellular Ig-like domains in an antiparallel manner, in which domain 1 of one PECAM-1 molecule binds to domain 1 (19) or domain 2 of the counter molecule of PECAM-1 (17, 18).

PECAM-1 has also been reported to mediate heterophilic binding to a number of non-PECAM-1 ligands. These include a heparin-containing proteoglycan (25, 28–30), the integrin αβ2 (29, 31), and CD38 (32). The binding sites in PECAM-1 responsible for interactions with two of these ligands, the putative proteoglycan and αβ2, were reported to be localized to regions in the second Ig-like domain (25, 29). Recently, however, the validity of some of these presumed ligand interactions has been challenged (18). Specifically, in assays that studied the binding of PECAM-1/Ig chimera, purified PECAM-1 in phospholipid vesicles, to PECAM-1-expressing cells, PECAM-1/αβ2, and PECAM-1/heparin interactions were not confirmed (18). Furthermore, in a subsequent detailed analysis, PECAM-1 was not found to bind heparin (33).

It is clear that some (but by no means all) of the differences may be due to the adhesion assay system used (cell-cell aggregation vs the binding of PECAM-1 constructs to cells). Each assay is admittedly artificial, with limitations, and each may in fact be looking at a different aspect of PECAM-1-dependent adhesion. We would note, however, for one animal model of neutrophil recruitment, the L cell aggregation assay appeared to correlate the best with in vivo observations. Specifically, for adhesion mediated by the PECAM/Ig chimera, all of the Abs (excluding mAb 35) blocked in vitro (homophilic) adhesion, yet two of the five Abs did not inhibit cutaneous inflammation, while none of the Abs that were active during in vivo inflammation blocked the aggregation of PECAM-1 L1/I2 transfectants. In contrast, the L cell aggregation assay identified two groups of Abs: one that was capable of blocking PECAM-1 homophilic aggregation and a second that exclusively blocked heterophilic aggregation. Only those Abs that were able to block homophilic aggregation inhibited cutaneous inflammation. Supporting this are the findings that mAb 1.3 (epitope includes residues 37–42 of domain 1; P. J. Newman and C. Padrock, unpublished observations), which does not effect PECAM-1-dependent L cell aggregation (34), and mAb 4G6 (epitope in domain 6; see Ref. 35), which blocks only heterophilic aggregation (34), also do not inhibit neutrophil accumulation in the human skin/SCID mouse chimera model (13 and H. DeLisser, unpublished observations). Currently, the one exception to the above is mAb Hec 7. This Ab, which has a complex epitope involving both domains 1 and 2 (16, 35), has been previously shown to inhibit cutaneous inflammation (13), but not PECAM-1-dependent L cell aggregation (34). mAb 35 differs from the other Abs in that although it promotes in vitro adhesion, it blocks cutaneous inflammation. The reasons for the inhibition observed in vivo are not clear, but may represent antagonism of PECAM-1 function due to inappropriate or excessive adhesion.

It has been previously noted that L cell transfectants expressing muPECAM-1 or huPECAM-1 when mixed together do not form mixed aggregates with each other, indicating that in the L cell system, muPECAM-1 and huPECAM-1 do not interact with each other (17). Thus, although the results of the L cell aggregation
### A. Presumed Location of the Binding Epitopes for mAbs 37, 51, 66

**Amino Acids 26-45**

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Sequences containing binding Epitopes
LTLQC or TLQC - 62; HVKP - 51; PQHQ - 37

### B. Presumed Location of the Binding Epitopes for mAbs 24, 35, and 40

**Amino Acids – 71-103**

|        | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 |
|--------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| **HUMAN** | E | V | R | I | Y | D | S | G | T | Y | K | C | T | V | I | V | N | N | K | E | K | T | T | A | Y | E | Q | L | L | V | E | G | V |
| **RAT** | q | a | R | v | f | h | a | G | k | Y | K | C | T | V | I | N | I | S | K | E | K | T | T | I | Y | E | Q | L | t | V | n | G | V |
| **BOVINE** | h | V | R | v | c | D | S | G | r | Y | K | C | n | V | I | n | N | l | g | e | T | T | p | Y | e | v | w | V | E | G | V |
| **MURINE** | q | V | R | v | f | h | S | G | k | Y | K | C | T | V | I | V | N | N | K | E | K | T | T | i | Y | e | v | k | V | h | G | V |

Sequences containing binding Epitopes
RIYD - 35; QLLV - 24 and 40

**FIGURE 9.** Identification of presumptive epitopes domain 1 anti-PECAM-1 Abs by cross species sequence comparisons. Shown are the amino acid sequences from different species of PECAM-1 for selected regions of the first Ig-like domain (aa 26–45 and 71–103). Indicated are the presumed binding epitopes for the six Abs studied. See text for details.

**FIGURE 10.** Modeling of the first Ig-like domain of PECAM-1. A model of the first Ig-like domain of PECAM-1 was developed to analyze the significance of the deduced locations of the anti-PECAM-1 domain Abs (see Materials and Methods). A, A ribbon diagram of the first Ig-like domain of PECAM-1, in which homologous sequences of murine and human PECAM-1 are in red and nonhomologous sequences are in white. B, Added to the ribbon diagram are the five acidic residues reported by Newton and associates (19) to be involved in homophilic adhesion. C, Overlaid on the ribbon diagram is the location of the residues containing the presumed epitopes for mAbs 37 and 51 (green), mAb 24 and 40 (magenta), mAb 62 (orange), and mAb 35 (yellow). D, Shown is the combined figure.
assay correlated the best with SCID mouse/human skin model of inflammation, these previous data suggest a possible limitation of L cell system. There is, however, evidence from immunostaining of human skin grafts on SCID mice that for murine vessels that have fused with human vessels, mouse and human PECAM-1 on the endothelium do in fact interact with each other (36, H. DeLisser, unpublished observations).

An important question, raised by the model that proposes that PECAM-1-dependent heterophilic adhesion is involved in in vivo leukocyte recruitment, is why heterophilic blocking Abs (mAbs 37, 51, and 4G6) do not also block neutrophil accumulation in the human skin/SCID mouse model? The lack of effect of these Abs on cutaneous inflammation is not surprising, however, if the species reactivity is considered. As these Abs do not bind muPECAM-1, they cannot bind murine neutrophils, and thus they would not be expected to impact on this step of the process that is proposed to involve only neutrophil PECAM-1.

Newton and associates (19) have recently identified two clusters of acidic residues (D11 and K89 and D35 and K50) that map to opposite faces of domain 1 and that appear to mediate PECAM-1-dependent homophilic binding. Given these findings and the results of the L cell aggregation studies, the location of the binding epitopes for our domain 1 Abs was compared with the position of these sequences using a computer-generated model of the structure of the first Ig-like domain of PECAM-1 (see the methods for the strategies employed in producing this model). From this model, a number of observations can be made about the Abs we studied (Fig. 10). The binding epitopes for our domain 1 Abs do not include any of these five residues. This is not unexpected given that our anti-human PECAM-1 Abs were generated in mice and thus only nonhomologous regions would be immunogenic. However, the epitopes of mAbs 62 and 24 and 40 are positioned such that Ab binding would be anticipated to shield two of these residues (D11 and D33), although their effects could still be mediated by distortions of the conformation of the molecule. In contrast, mAbs 37 and 51 bind to sequences that are remote from any of these five acidic residues and thus, not surprisingly, Ab binding did not inhibit L-cell-dependent homophilic aggregation. Ab 35 is intriguing in that its binding epitope is close to residues D51 and K50, but its binding in fact augments rather than inhibits adhesion. This suggests that this Ab may alter the conformation of the molecule in a way that makes these residues more accessible for binding to other PECAM-1 molecules. Finally, the Ab cross-blocking studies indicate that the epitopes for these two Abs are located more closely to each other than is suggested by our computer-generated model.

In conclusion, these studies provide further evidence of the role of PECAM-1 in in vivo inflammation and suggest that antagonists of PECAM-1 that target the first domain of PECAM-1 may provide the basis for the development of new anti-inflammatory agents.

Acknowledgments

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