

Molecular and Cellular Properties of PECAM-1 (endoCAM/CD31): A Novel Vascular Cell–Cell Adhesion Molecule

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Abstract. PECAM-1 is a 130–120-kD integral membrane glycoprotein found on the surface of platelets, at endothelial intercellular junctions in culture, and on cells of myeloid lineage. Previous studies have shown that it is a member of the immunoglobulin gene superfamily and that antibodies against the bovine form of this protein (endoCAM) can inhibit endothelial cell–cell interactions. These data suggest that PECAM-1 may function as a vascular cell adhesion molecule. The function of this molecule has been further evaluated by transfecting cells with a full-length PECAM-1 cDNA. Transfected COS-7, mouse 3T3 and L cells expressed a 130–120-kD glycoprotein on their cell surface that reacted with anti-PECAM-1 poly-

clonal and monoclonal antibodies. COS-7 and 3T3 cell transfectants formed cell–cell junctions that were highly enriched in PECAM-1, reminiscent of its distribution at endothelial cell–cell borders. In contrast, this protein remained diffusely distributed within the plasma membrane of PECAM-1 transfected cells that were in contact with mock transfectants. Mouse L cells stably transfected with PECAM-1 demonstrated calcium-dependent aggregation that was inhibited by anti-PECAM antibodies. These results demonstrate that PECAM-1 mediates cell–cell adhesion and support the idea that it may be involved in some of the interactive events taking place during thrombosis, wound healing, and angiogenesis.

CELL–CELL interactions are mediated by at least four distinct families of transmembrane glycoproteins: integrins, cadherins, selectins (Lec-CAMs),¹ and cell adhesion molecules belonging to the immunoglobulin superfamily (reviewed in Albelda and Buck, 1990). The integrin family is a broadly distributed group of receptors composed of homologous, noncovalently associated α/β heterodimer pairs that mediate leukocyte–leukocyte and leukocyte–endothelial cell adhesion (Carlos and Harlan, 1990; Osborn, 1990; Springer, 1990), as well as cellular interactions with extracellular matrix components such as collagen, laminin, fibrinogen, and fibronectin (Buck and Horwitz, 1987; Ruoslahti and Piersbacher, 1987; Hynes, 1987). The cadherin family is a developmentally regulated group of structurally related, single chain 120–130-kD glycoproteins that mediate calcium-dependent cellular adhesion in a homophilic and tissue-specific manner (Takeichi, 1988, 1991). The selectins or Lec-CAMs (including ELAM-1, GMP-140, and gp90^{MEL-14}) bind leukocytes to endothelial cells via a lectin-like domain at the amino terminus of the molecule (Bevilacqua et al., 1989; Johnston et al., 1989; Larsen et al., 1989; Butcher et al., 1990; Imai et al., 1990; Bradley et al., 1990).

1. *Abbreviations used in this paper:* LecCAM, selectin cell adhesion molecule; PECAM, platelet/endothelial cell adhesion molecule.

The cell adhesion molecules (CAMs) belonging to the immunoglobulin gene (Ig) superfamily show diversity in function and tissue distribution. Like other members of the Ig-superfamily, CAMs share a common structure, the immunoglobulin homology unit, that is characterized by an amino acid sequence ~ 100 amino acids in length, having a centrally placed disulfide bridge that stabilizes a series of antiparallel β -strands into the so-called antibody fold (Hunkapiller and Hood, 1989). The immunoglobulin superfamily CAMs are thought to have wide-ranging functions, and participate in a variety of homophilic and heterophilic cellular interactions (Williams and Barclay, 1988), including those that take place during development (N-CAM) (Cunningham et al., 1987; Edelman, 1988), inflammation and wound healing (ICAM-1, VCAM-1) (Wawryk et al., 1989; Elices et al., 1990), and possibly oncogenesis (CEA: colon carcinoma cell metastasis) (Benchimol et al., 1989).

PECAM-1 (platelet/endothelial cell adhesion molecule-1) represents a recently characterized member of the immunoglobulin superfamily that is found on the surface of platelets, some leukocytes and at endothelial cell intercellular junctions in culture (Muller et al., 1989; Newman et al., 1990). A homologous glycoprotein, “endothelial cell adhesion molecule” (endoCAM) has been described on bovine endothelial cells and platelets and has been shown to mediate endothelial

cell-cell interactions (Albelda et al., 1990). Immunohistochemical and biochemical studies (Albelda et al., 1990; Newman et al., 1990) indicate that PECAM-1 is identical to the *hec7* and CD31 antigens that have been described on human blood platelets, monocytes, and neutrophils, as well as on large and small vessel endothelial cells (Muller et al., 1989; van Mourik et al., 1985; Stockinger et al., 1990). Bone marrow stem cells and transformed cell lines of the myeloid and megakaryocytic lineage also express CD31 (Ohto et al., 1985; Goyert et al., 1986; Cabanas et al., 1989; Simmons et al., 1990).

Although the precise functions of PECAM-1 (CD31) have not been determined, we have shown (a) that PECAM-1 is concentrated on cultured endothelial cells at intercellular junctions as they come to appose one another (Muller et al., 1989; Albelda et al., 1990); (b) that PECAM-1 is constitutively expressed on continuous, but not sinusoidal, endothelium *in situ* (Muller et al., 1989; Albelda et al., 1990); and (c) that antibodies against endoCAM, the bovine equivalent of PECAM-1, inhibit the ability of endothelial cells to form a confluent monolayer (Albelda et al., 1990). These data indicate that this molecule is involved in endothelial cell-cell adhesion.

The homology of PECAM-1 to other CAMs of the immunoglobulin superfamily, its presence on platelets, white cells, and endothelium, its localization to endothelial cell junctions, and the initial functional data described above, all suggest that PECAM-1 is an important vascular cell adhesion molecule. In this manuscript, we present the full-length cDNA sequence, describe the expression of PECAM-1 in heterologous COS-7, 3T3, and L cells, and demonstrate that PECAM-1 is capable of mediating the aggregation of stably transfected mouse L cells. These experiments directly demonstrate the role of PECAM-1 in adhesion events.

Materials and Methods

Antibodies

The following antibodies were used: *hec7* and WM59, mAbs specific for human PECAM-1 (Muller et al., 1989; Newman et al., 1990); a polyclonal antibody directed against bovine endoCAM that cross reacts with human PECAM-1 (Albelda et al., 1990); an anti-CD31 monoclonal antibody generously provided by Jan Sixma (University Hospital, Utrecht, the Netherlands); a polyclonal anti-human PECAM-1 antibody; and an antiserum to von Willebrand factor (vWF) (Calbiochem-Behring Diagnostics, La Jolla, CA). FITC-conjugated anti-mouse and anti-rabbit antisera were purchased from Organon Technika Corp (West Chester, PA).

The polyclonal anti-PECAM antiserum was produced using the same techniques as were used to prepare an anti-endoCAM antiserum (Albelda et al., 1990). PECAM-1 was purified from outdated human platelets by affinity chromatography using the anti-PECAM mAb 1.3 (IgG1 subclass) and 100- μ g aliquots of purified protein were injected into a rabbit in combination with Freund's adjuvant at 2-wk intervals for a total of four injections. Purified immunoglobulin was isolated by protein A (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography. The specificity of the polyclonal antiserum was equivalent to that of the monoclonal antibodies (see below).

Cells

Human umbilical vein endothelial cells were isolated and cultured in medium 199 containing 15% FBS, 75–100 μ g/ml endothelial cell growth factor, 100 μ g/ml heparin, and 2 mM *L*-glutamine (Albelda et al., 1989). COS-7, 3T3 cells, and mouse L cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). COS-7 cells and 3T3 cells were cultured in DMEM with 10% FBS. L cells were cultured in RPMI medium with 10% FBS.

Nucleotide Sequencing

The complete nucleotide sequence of PECAM-1 was determined from three overlapping λ gt11 phage clones (Newman et al., 1990). A series of overlapping nested deletions were generated using exonuclease III. After this digestion, the remaining single-stranded material was cleaved with S1 nuclease, the ends made blunt with T4 DNA polymerase, and the plasmids resealed with T4 DNA ligase. Both strands of all overlapping clones were sequenced by the dideoxy-chain termination method using Sequenase DNA polymerase (United States Biochemical Co., Cleveland, OH).

Northern Blot Analysis

Total cellular RNA was prepared from both human umbilical vein endothelial cells and HEL cells using the method of Chirgwin et al. (1979) and the poly (A⁺) fraction purified on Oligo d(T) cellulose according to the procedure of Avril and Leder (1972). 15 μ g of total RNA or 3 μ g of mRNA was denatured in 1 M glyoxal in the presence of 50% DMSO, electrophoresed through 1% agarose, and transferred to nitrocellulose according to the procedure of Thomas (1980). Both prehybridization and hybridization were performed at 42°C in a buffer containing 40% formamide, 4 \times SSC (SSC=0.15 M NaCl, 15 mM trisodium citrate, pH 7.0), 7 mM Tris pH 7.4, 1 \times Denhardt's solution, and 50 μ g/ml of salmon sperm DNA. Filters were washed at high stringency, with the final two washes performed at 68°C in 0.1% SSC containing 1.0% SDS. Northern blots were also performed using Genescreen Plus (Dupont New England Nuclear, Wilmington, DE) according to the manufacturer's directions.

Transfection of COS-7 Cells

A 2.56-kb cDNA fragment containing the entire coding sequence for PECAM-1 was subcloned from PEGEM 7Z (Promega Biotec, Madison, WI) into the HindIII cloning site of the SV40 expression vector pESP-SVTEXP (Reddy and Rao, 1986; Solowska et al., 1989). The resulting plasmid was designated PECAM/TEX. COS cell transfections were performed by calcium phosphate-DNA coprecipitation (Sambrook et al., 1989). Confluent T75 flasks of COS-7 cells were split 1:15 and plated on 100-mm petri dishes in DMEM with 10% FBS. After 24 h, the cells were transfected with 6 μ g PECAM/TEX cDNA in combination with 17 μ g of Bluescript plasmid DNA (Stratagene Corp., La Jolla, CA). After incubation at 37°C for 4 h, the cells were washed with PBS, and fresh DMEM/10% FBS was added. The plates were maintained for an additional 36 h before extraction or immunofluorescence staining.

Transfection of 3T3 and L Cells

To establish cells stably expressing PECAM-1 cDNA, NIH 3T3 cells were transfected as previously described (Solowska et al., 1989). Cells were plated at 1×10^5 per 100-mm petri dish in DMEM with 10% FBS. After 24 h, the cells were cotransfected with 6 μ g of the PECAM/TEX plasmid in combination with 100 ng pSV2neo and 17 μ g calf thymus DNA as a calcium phosphate precipitate. Cells were incubated for 20–22 h, washed with PBS, and fresh medium was replaced. 2 d later, the transfected cells were split 1:25 to 1:50 and incubated in DMEM supplemented with 10% FBS and 1 mg/ml G418 (Geneticin; Gibco Laboratories, Grand Island, NY). After \sim 2 wk, the G418-resistant clones were isolated and expanded. The 3T3 clones expressing PECAM-1 were identified by indirect immunofluorescence staining using the PECAM-1 specific mAb *hec7*. The most positively staining clones were then subcloned by limiting dilution and a cell line expressing PECAM-1 on \sim 80–90% cells was used for further studies.

Mouse L cells were transfected in a similar manner as the 3T3 cells with the following changes: (a) the pH of the transfection buffer was adjusted to 6.95 rather than 7.2; (b) cells were plated at 5×10^5 per 100 mm petri dish; and (c) clones were selected in RPMI media supplemented with 10% FBS and 0.5 mg/ml G418. After selection by immunofluorescence, the most positive clones were further characterized by FACS analysis (see below) using the anti-PECAM mAb *hec7*.

Flow Cytometry

L cells transfected with pSV2neo alone or PECAM/TEX were nonenzymatically removed from T25 flasks (see below), washed in PBS/4% BSA, and treated with anti-PECAM-1 mAb for 1 h at 4°C. The primary antibody was removed, the cells washed twice with ice-cold PBS, and a 1:200 dilution of FITC-labeled goat anti-mouse secondary antibody added for 1 h at 4°C. After washing in cold PBS, flow cytometry was performed using an Ortho

Cytofluorograph 50H cell sorter equipped with a 2150 Data Handling System (Ortho Instruments, Westwood, MA). Fluorescence intensity was expressed as arbitrary units on a log scale.

Cell Labeling

To metabolically label cultured cells, confluent flasks were washed with PBS, incubated in methionine-free DMEM supplemented with 10% dialyzed FBS for 1 h, and then labeled with [³⁵S]methionine (New England Nuclear, Boston, MA) (200 μ Ci for 25 cm², 300 μ Ci for 75-cm² flasks) in fresh medium. For labeling of human endothelial cells, the methionine-free DMEM/10% FBS was supplemented with endothelial cell growth factor and heparin as described (Albelda et al., 1989). After 24 h, the cells were washed with PBS and extracted as described below.

Cultured cells were surface labeled with ¹²⁵I using previously described techniques (Albelda et al., 1989). Briefly, transfected COS cells were washed three times with PBS and exposed to carrier free ¹²⁵I (1 mCi/100-mm plate) in the presence of lactoperoxidase, glucose oxidase, and glucose. After labeling for 30 min, the reaction was terminated by the addition of free iodine and the cells were extracted. Membrane extracts were prepared by adding small amounts (i.e., two or three times the volume of the cell pellet) of TNC (0.01 M Tris acetate, pH 8.0, 0.5% NP-40, 0.5 mM CaCl₂) with 2 mM PMSF to the pellet, pipetting on ice for 15 min, and then centrifuging for 30 min at 12,000 g. The resulting supernatant was used immediately or frozen at -20°C until used.

Immunoprecipitation

Nonionic detergent extracts were preadsorbed for 30 min at 4°C with Protein A conjugated to Sepharose beads (Pharmacia Fine Chemicals). After removal of the beads, the appropriate antibody was added to the precleared extract for 1 h at 4°C. Immunocomplexes were collected by precipitation with fresh Protein A-sepharose beads for 1 h at 4°C, washed five times with a buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 0.1% SDS. The sample was then dissolved in electrophoresis sample buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, pH 6.8), electrophoresed on 6% polyacrylamide gels and processed for autoradiography as described below.

One-Dimensional Gel Electrophoresis

Samples were analyzed by SDS-PAGE using 6% polyacrylamide gels by the method of Laemmli (1970) without the use of reducing agents. Gels were dried and exposed to Kodak XR-5 X-ray film at -70°C.

Immunofluorescence

Endothelial cells were plated at 4 × 10⁴ cells/cm² on glass coverslips coated with 1% gelatin in PBS (Difco, Detroit, MI). After the cells had grown to confluence, fixation and staining was performed using previously described methods (Albelda et al., 1989). Briefly, cells were fixed with 3% paraformaldehyde for 20 min and then permeabilized with ice-cold 0.5% NP-40 for 1 min. After extensive washing, 50 μ l of antibody was added for 1 h. After rinsing, the coverslips were stained with 50 μ l of a 1:200 dilution of FITC-labeled antiserum antibodies for 1 h. Cells were viewed on a Zeiss phase-epifluorescent microscope using a 63× planapochromat oil-immersion lens numerical aperture 1.4 and photographed using Tri-X film at 400 ASA.

COS-7 cells were plated on gelatin-coated glass coverslips placed in six-well plates. 1 d after plating, the cells were transfected with PECAM-1. 48 h after transfection, the cells were fixed, stained, and analyzed as described above.

For coculture experiments, equal numbers of endothelial cells and transfected 3T3 cells were mixed and plated together to achieve a final cell density of 2 × 10⁴ cells/cm² on glass coverslips that had been coated with 10 μ g/ml of purified plasma fibronectin (New York Blood Center, New York, NY) for 1 h. After overnight incubation in endothelial cell medium, the coverslips were fixed, permeabilized, and stained with both anti-PECAM-1 mAb (hec7) and rabbit anti-vWF antiserum. After washing, the cells were treated with fluorescein-conjugated anti-mouse antibody and rhodamine-conjugated anti-rabbit antibody. Control experiments demonstrated no cross-species reactivity between the second antibody reagents.

Aggregation of L Cell Transfectants

A variation of the aggregation assay of Takeichi (1977) established for the study of cells expressing cadherin molecules was developed. Stable L cell transfectants were washed twice in HBSS without divalent cations and then

incubated for 10–20 min at 37°C in 5 mM EGTA in HBSS, pH 7.4. When inverted phase-contrast microscopy showed that the cells had visibly rounded and begun to detach, they were removed from the dish by gentle pipetting. The cells were washed twice in cold HBSS. In some experiments, cells were incubated with 25 μ g/ml of purified anti-PECAM-1 polyclonal antibodies or nonimmune rabbit IgG at this point. In these experiments cells were then resuspended to ~10⁶ cells/ml and incubated with antibodies on ice for 20 min. Cells were subsequently washed twice in cold HBSS to remove unbound antibody and resuspended to ~10⁶ cells/ml in HBSS (37°C) with or without 1 mM CaCl₂. Cells were monodispersed and viability was >95%, as assessed by Trypan blue dye exclusion.

1-ml aliquots were transferred to wells in a 24-well tissue culture tray (Costar Corp., Cambridge, MA) that had been previously incubated with 1% HSA in HBSS for at least 1 h and washed thoroughly with HBSS immediately before use. This treatment prevents nonspecific cell sticking to the tissue culture dish (Takeichi, 1977). The tissue culture trays containing the suspended L cells were rotated on a gyratory shaker (90 rpm) at 37°C. At desired intervals, aggregation was stopped by addition of glutaraldehyde to a final concentration of 2%.

Aggregation was quantified by examining representative aliquots of equal volumes from each sample on a hemacytometer grid under phase contrast optics. The number of cells remaining as single cells or present in aggregates (\geq three cells) were counted in nine squares. At least 800 cells were counted for each sample. Data are expressed as the percent of total cells present in aggregates.

Results

Nucleotide Sequence and Message Size of PECAM-1

To more fully characterize the structure and function of PECAM-1 and to prepare a full-length expression construct, its nucleotide sequence was determined from two overlapping clones. As shown in Fig. 1, the 2,557-bp sequence contains a 141-bp 5' untranslated (UT) region, an open reading frame of 2,214 bp that encodes 738 amino acids (AA), and a 202-bp 3' UT region. The ensuing 3' UT region has apparently been only partially determined, since Northern blot analysis (Fig. 2) indicates that the full-length mRNA from either HEL cells or endothelial cells was ~4.2 kb. A smaller mRNA species at ~3.8 kb was also consistently observed under high stringency hybridization and wash conditions, however its significance is as yet unknown.

Expression of PECAM-1 in COS-7 and 3T3 Cells

To gain insight into the nature of PECAM-mediated cellular recognition events and to confirm the validity of our cDNA sequence, a full-length PECAM-1 cDNA was cloned into the expression vector pESP-SVTEXP and transfected into COS and 3T3 cells.

PECAM-1 expression was demonstrated by immunoprecipitation of [³⁵S]methionine-labeled extracts from transfected cells and human umbilical vein endothelial cells using an anti-PECAM-1 monoclonal antibody (Fig. 3, top). Protein bands at 130 and 120 kD were detected in transfected 3T3 cells (Fig. 3, lane B) and COS cells (Fig. 3, lane C) that behaved identically to the material immunoprecipitated from control endothelial cells (Fig. 3, lane A). The lower band likely represents a precursor protein as it was not seen when surface-labeled material was immunoprecipitated (see Fig. 3, E–G). No cross-reacting material of similar size was detected in control 3T3 cells (Fig. 3, lane D). Since the cDNA itself encodes a polypeptide core of only 80 kD (Newman et al., 1990 and Fig. 1), these data suggest that the primary sequence of recombinant PECAM-1 is sufficient to direct normal or near-normal posttranslational processing in monkey kidney cells or murine fibroblasts.

GAATTCGGGAGAAGTGACCAGAGCAATTTCTGCTTTTACAGGGCGGGTTTCTCAACGGTGACTGTGGGCAGTGCCTTCTGCTGAGCGAGTATGGCCCAAGGCAGAACTAACTGTG 120

-27

CCTGCAGTCTTCACTCTCAGGATGCAGCCGAGGTGGGCCAAGGGGCCAGATGGCTTGGAGTCTGCTGACCCTTCTGCTCTGTTCAAGCCTTGGGGTCAAGAAAACCTTTTCA 240
M Q P R W A Q G A T M W L G V L L L T L L L L C S S L E G Q E N S F T 6

ATCAACAGTGTGACATGAAGAGCCTGCCGGACTGGACGGTGCAAAATGGGAAGAACCTGACCCTGCAGTGETTCGCGGATGTCAGCACCCCTCTCAGTCAAGCCTCAGCCAGATG 360
 I N S V D M K S L P D W T V Q N G K N L T L Q © F A D V S T T S H V K P Q H Q M 46

CTGTCTATAAGGATGACGTGCTGTTTTACAACATCTCCTCCATGAAGAGCACAGAGATTATTTTATCTGAAAGTCCGGATCTATGACTCAGGGACATATAAATGACTGTGATTGTG 480
 L F Y K D D V L F Y N I S S M K S T E S Y F I P E V R I Y D S G T Y K © T V I V 86

AACAACAAGAGAAAACCACTGCAGAGTACCAGCTGTTGGTGGAAAGGAGTCCCAAGTCCAGGGTGCACACTGGACAAGAAAGAGGCCATCCAAGTGGGATCGTGAGGGTCAACTGTTCT 600
 N N K E K T T A E Y Q L L V E G V P S P R V T L D K K E A I Q G G I V R V N © S 126

GTCCAGAGGAAAAGGCCCAATACACTTCACAATTGAAAACCTGAACTAAATGAAAACCTGGTCAAGCTGAAAAGAGAGAAAGCAATTCGAGACCAGAAATTTGTGATCTGCAATTC 720
 V P E E K A P I H F T I E K L E L N E K M V K L K R E K N S R D Q N F V I L E F 166

CCCCTTGGAGAACAGGACCGCGTTTTATCCTTCCGATGTCAAGCTAGGATCATTCTGGGATCCATATGCAGACCTCAGAATCTACCAAGAGTGAACCTGGTACCCTGACGGAACTCCTTC 840
 P V E E Q D R V L S F R © Q A R I I S G I H M Q T S E S T K S E L V T V T E S F 206

TCTACACCAAGTCCACATCAGCCCCACCGAATGATCATGGAAGGAGCTCAGCTCCACATTAAGTGACCATTAAGTGCACCTCAAGTGCACCTGGCCCAGGAGTTTCCAGAAATCATAATCAG 960
 S T P K F H I S P T G M I M E G A Q L H I K © T I Q V T H L A Q E F P E I I I Q 246

AAGGACAAGCGGATTTGGCCCAACAGACATGGCAACAAGGCTGTGACTCAGTGCAGTCCAGTGGTGGAGCAGTGGCAACTACAGTGCAAAGTGGAGTCCAGCCGCATATCCAAG 1080
 K D K A I V A H N R H G N K A V Y S V M A M V E H S G N Y T © K V E S S R I S K 286

GTCAGCAGCATCGTGGTCAACATAACAGAATTTTTCCAAGCCCAAGTGAATCTTCTTCCACACATCTGGACCAAGGTGAAAGACTGAACCTGTCTGCTCCATCCAGGAGCACCT 1200
 V S S I V V N I T E L F S K P E L E S S F T H L D Q G E R L N L S © S I P G A P 326

CCAGCCAACCTCACCATCCAGAAGGAGATACGATTGTGTACAGACTCAAGATTTCCCAAGATAGCCTCAAAGTCCGACAGTGGGACGTATATCTGCACTGCAGGTATTGACAAAGT 1320
 P A N F T I Q K E D T I V S Q T Q D F T K I A S K S D S G T Y I © T A G I D K V 366

GTCAGAAAAGCAACACAGTCCAGATAGTCGATGTGAAATGCTCTCCAGCCAGGATTTCTTATGATGCCAGTTTGGAGTATAAAAGGACAGCCATCGAAGTCCGTTGCGAATCG 1440
 V K K S N T V Q I V V C E M L S Q P R I S Y D A Q F E V I K G Q T I E V R © E S 406

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 I S G T L P I S Y Q L L K T S K V L E N N S T K N S N D P A V F K D N P T E D V E 446

TACCAAGTGTGTCAGATAATGCCATCCCATGCCAAAATGTTAAGTGAAGTCTGAGGGTGAAGGTGATAGCCCGGTGGATGAGGTCAGATTTCTATCTGTCAGTAAAGTGGT 1680
 Y Q © V A D N C H S H A K M L S E V L R V K V I A P V D E V Q I S I L S S K V V 486

GAGTCTGGAGAGGACATTTGCTGCAATGTGCTGTGAATGAAGGATCTGGTCCATCACCTATAAGTTTTACAGAGAAAAGAGGGCAAACCCCTTCTATCAATGACCTCAAATGCCACC 1800
 E S G E D I V L Q © A V N E G S G P I T Y K F Y R E K E G K P F Y Q M T S N A T 526

CAGGCATTTTGGACCAAGCAGAAGGCTAGCAAGGAACAGGAGGAGATTAAGTCTGACAGCCTTCAACAGAGCCAACCAGCCTCCAGTGTCCCCAGAAGCAAAATGACAGTGCAGA 1920
 Q A F W T K Q K A S K E Q E G E Y Y © T A F N R A N H A S S V P R S K I L T V R 566

GTCATCTTCCCCATGGAAGAAAGGACTTATTGCAGTGGTATCATCGGAGTATCATTGCTCTCTTGTATCATTGCGGCCAAATGTTATTTTCTGAGGAAAGCCAAAGCCAAAGCAGATG 2040
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 M K P I N D N K E P L N S D V Q Y T E V Q V S S A E S H K D L G K K D T E T V Y 686

AGTGAAGTCCGAAAGCTGTCCCTGATGCCGTGGAAGCAGATACTCTAGAAGGAGGCTCCCTTGTGGAAGT**TAG**CAGCAAGGCCAGATGCACATCCCTGGAAGGACATCCATGTT 2400
 S E V R K A V P D A V E S R Y S R T E G S L D G T 711

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GCTAAGTTAATGTTGGGTAGAAGAGATACAGAGGGG 2557

Figure 1. Primary structure and nucleotide sequence of PECAM-1. The amino acid sequence is numbered from the first residue after the predicted cleavage site of the 27-amino acid signal peptide. The hydrophobic putative signal peptide and transmembrane sequence are underlined. Two naturally occurring EcoRI sites are boxed. Cysteine residues thought to participate in disulfide bond formation within individual immunoglobulin homology units are circled. The sequence contains nine potential *N*-linked glycosylation sites, denoted by closed triangles, and one potential tyrosine phosphorylation site at residue 686, indicated with a closed circle. The TAG stop codon is underlined and in bold type. These sequence data are available from EMBL/GenBank/DBJ under accession number M28526.

To determine if the protein was directed to the plasma membrane of the transfected cells and to further confirm the relationship of PECAM-1 to endoCAM and the CD31 antigen, extracts from ¹²⁵I-surface-labeled PECAM-1 trans-

fectected or sham transfected COS-7 cells were immunoprecipitated with an anti-endoCAM polyclonal antibody (Fig. 3 E), an anti-CD31 mAb (Fig. 3 F), or an anti-PECAM-1 monoclonal antibody (Fig. 3 G). A 130-kD protein was clearly

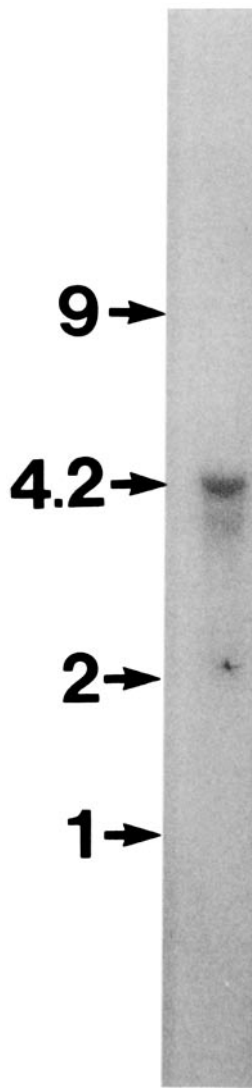


Figure 2. Northern blot analysis of PECAM-1. Total cellular RNA was isolated from human umbilical endothelial cells, denatured with glyoxal, and separated on a 1% agarose gel. After transfer to nitrocellulose, the blot was hybridized with a nick-translated PECAM-1 cDNA probe under high-stringency conditions (see Materials and Methods). Size markers in kilobases are shown on the left. Under these conditions, a major RNA at 4.2 kb and a smaller band at 3.8 kb are revealed.

identified on the surface of transfected COS-7 cells with all three antibodies (*arrow*) confirming the identity of CD31, PECAM-1, and endoCAM suggested earlier (Albelda et al., 1990; Newman et al., 1990).

Transfected PECAM-1 Protein Is Concentrated at the Intercellular Junctions of Adjacent Transfected Cells

When human or bovine endothelial cells were stained with anti-PECAM-1 or endoCAM antibodies, a striking localization to cell-cell borders was observed (Muller et al., 1989; Albelda et al., 1990). It was therefore necessary to examine the cellular localization of PECAM-1 in the transfected cells. To accomplish this, COS-7 cells, transfected with PECAM-1, were plated onto glass coverslips. After 48 h, the cells were fixed, permeabilized, and stained with anti-PECAM-1 mAb followed by a secondary FITC-labeled anti-mouse antibody. In individual transfected cells that were not in contact with other transfected cells, a bright, diffuse pattern of fluorescence was observed (data not shown). In contrast, when transfected cells were in contact with other transfected cells, the PECAM-1 antigen localized to cell-cell borders (Fig. 4 B) in a manner very similar to endothelial cells (Fig. 4 A).

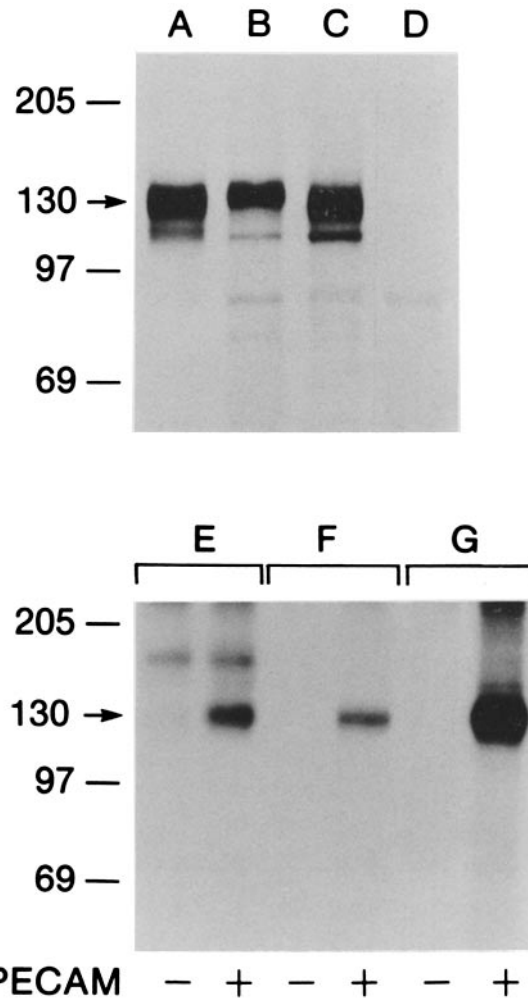


Figure 3. Expression of PECAM-1 in COS-7 and 3T3 cells. (*Top*) Nonionic detergent extracts from cells metabolically labeled with [³⁵S]methionine were immunoprecipitated with an anti-PECAM-1 mAb. Lane A, human umbilical vein endothelial cells; lane B, PECAM-1-transfected 3T3 cells; lane C, PECAM-1 transfected COS-7 cells; lane D, neomycin-transfected 3T3 cells. Transfected cells expressed 130- and 120-kD proteins (*arrow*) similar in size to those expressed by human umbilical vein endothelial cells. (*Bottom*) Extracts from ¹²⁵I-surface-labeled COS-7 cells that had been transfected with PECAM-1 cDNA (+) or sham transfected (-) were immunoprecipitated with three different antibodies. Lanes E, anti-bovine endoCAM polyclonal antiserum; lanes F, anti-CD31 mAb; lanes G, *hec7*, an anti-human PECAM-1 mAb. A 130-kD protein (*arrow*) was recognized in transfected cells by all three antibodies confirming the relationship among these molecules. Molecular weight markers in kilodaltons are shown on the left.

When a transfected cell contacted a nontransfected cell (Fig. 4 B, *arrowheads*), there was no cell-cell localization, despite the fact that the same transfected cell did localize PECAM-1 to the cell-cell border of an adjacent transfected cell.

To determine if transfected PECAM-1 protein was able to interact with its natural counterreceptor on human endothelial cells, PECAM-1 transfected 3T3 cells were co-cultured with human umbilical vein cells. Fig. 5 shows a double-label immunofluorescence experiment in which PECAM-1 expression was detected using an mAb (Fig. 5 A) and the endothelial cells within the culture were identified using a polyclonal

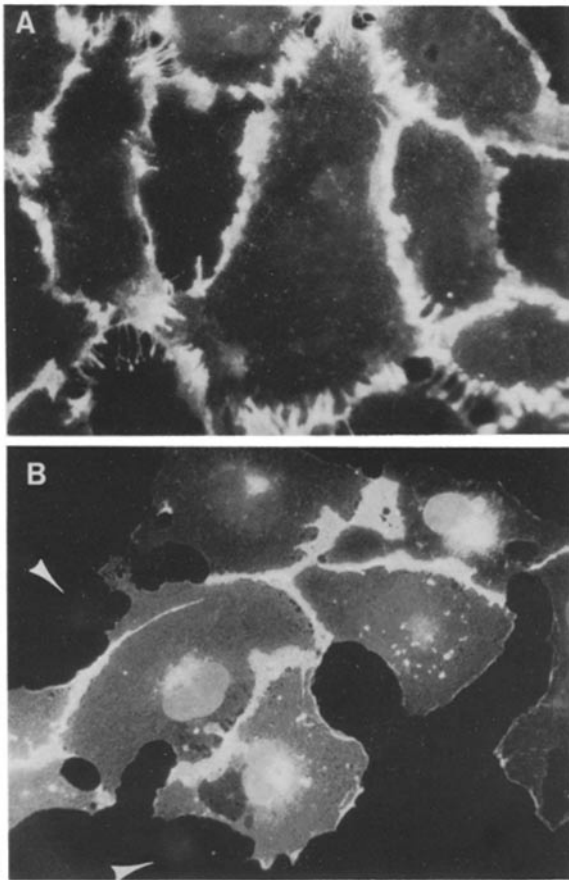


Figure 4. Transfected PECAM-1 protein concentrates at the intercellular junctions of adjacently transfected COS-7 cells. Human umbilical vein endothelial cells (A) or PECAM-1 transfected COS-7 cells (B) were fixed, permeabilized, and stained with an anti-PECAM-1 mAb followed by a secondary FITC-labeled anti-mouse antibody. The endothelial cells show the characteristic concentration of protein at intercellular borders (A). Adjacent transfected cells show a similar pattern of intercellular staining (B). In contrast, when non-transfected cells (arrows) contact transfected cells, there is no localization of PECAM-1 protein at the cell-cell border. These findings suggest that PECAM-1 functions in a homophilic manner.

rabbit antiserum specific for vWF that is not expressed by the 3T3 cells in the same culture (Fig. 5 B). The vWF-positive endothelial cells (Fig. 5 B, arrows) were readily distinguishable from the transfected 3T3 cells (Fig. 5 B, arrowheads). PECAM-1 is seen concentrated between apposing endothelial cells (Fig. 5 A, solid arrowhead) and apposing transfected 3T3 cells (Fig. 5 A, open arrowhead). In addition, PECAM-1 can be found localized at points of contact between the endothelial cells and transfected 3T3 cells (Fig. 5 A, arrow). No such localization of PECAM-1 was seen at the junction of non-transfected 3T3 cells and human endothelial cells (data not shown). Thus, transfected PECAM-1 appears to recognize its ligand on endothelial cells in the same manner as endogenous endothelial PECAM-1.

PECAM-1 Mediates the Aggregation of Transfected L Cells

To directly evaluate the ability of PECAM-1 to mediate adhe-

sion, cell aggregation studies were performed. Mouse L cell fibroblasts do not spontaneously aggregate with each other (Takeichi, 1977). However, L cells expressing adhesion molecules such as various cadherins (Nose et al., 1988) or CD44 (St. John et al., 1990) have been shown to aggregate specifically. To use this system to test whether PECAM-1 was capable of mediating cell-cell adhesion, we stably transfected L cells with PECAM cDNA that had been inserted into our eukaryotic expression vector, pESP-SVTEXP. FACS analysis showed that 65% of the transfected L cells expressed significant amounts of the protein on their surfaces (Fig. 6 b).

Non-enzymatically resuspended L cells expressing PECAM-1 rapidly formed aggregates composed of three to several dozen cells (Fig. 6 d) while cells transfected with the neomycin gene only did not (Fig. 6 c). Distinct aggregation was evident within 5 min (data not shown) and nearly maximal levels were achieved by 15 min (Fig. 6 a). Cell viability was essentially unchanged over the course of the assay. The aggregation of the PECAM-1 transfectants required the presence of calcium. The rate and extent of aggregation of the PECAM-1 transfectants was similar to those reported for L cells transfected with the other adhesion molecules cited above.

The specificity of the aggregation reaction was tested using a polyclonal antibody prepared against purified human PECAM-1 (Fig. 7). This reagent reacted only with PECAM-1 as assessed by immunoprecipitation of [³⁵S]methionine-labeled cells (Fig. 7). The polyclonal antiserum reacted with the same material in extracts of endothelial cells as did the anti-PECAM mAb (Fig. 7, A and C). No reactivity was noted with preimmune serum from the same rabbit (Fig. 7 B). Similarly, the polyclonal anti-PECAM-1 antiserum immunoprecipitated a doublet of the same relative molecular mass from the transfected L cells used in the aggregation assay (Fig. 7 E). This protein was not detected in nontransfected L cells (Fig. 7 G). No material was immunoprecipitated from transfected (Fig. 7 D) or nontransfected L cells (Fig. 7 F) with preimmune serum.

Fig. 8 demonstrates the effect of this antibody on PECAM-1 mediated L cell aggregation. As noted in Fig. 6, only cells transfected with the vector carrying PECAM-1 participated in aggregate formation which was complete by 30 min. Exposure of cells to the polyclonal PECAM-1 antibody reduced aggregation to the background levels noted with cells transfected with the neomycin vector alone (Fig. 8). Thus, the aggregation noted in transfected L cells is directly mediated by PECAM-1 expressed on the surface of these cells.

Discussion

The ability of circulating blood cells to adhere to one another and to the vascular endothelium is a highly regulated process fundamental to immunity, inflammation and thrombosis. These cell-cell interactions are mediated by a variety of specific adhesion molecules (reviewed by Butcher, 1989; Albelda and Buck, 1990; Carlos and Harlan, 1990; and Springer, 1990). Information about these molecules and the mechanisms of their adhesive interactions is critical to an understanding of the processes in which they are involved. PECAM-1 (CD31) is the newest of the vascular cell adhesion molecules to be reported.

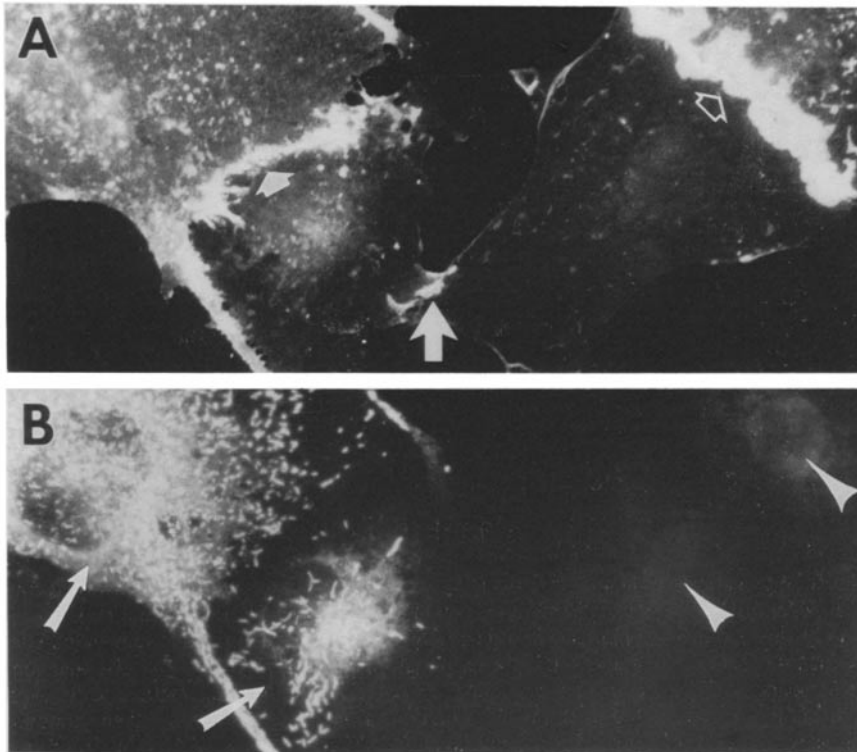


Figure 5. Transfected PECAM-1 protein can interact with native PECAM-1 protein on human endothelial cells. 3T3 cells transfected with PECAM-1 cDNA were co-plated with human umbilical vein endothelial cells and simultaneously stained with an anti-PECAM-1 monoclonal antibody (*A*) and an anti-von Willebrand's factor (vWF) polyclonal antiserum (*B*). Identical fields containing four cells are shown in *A* and *B*. The cells were then treated with FITC-labeled anti-mouse antibody (*A*) and rhodamine-labeled anti-rabbit antibody (*B*). Four cells can be visualized. The reactivity with vWF antibody (*B*, *solid arrows*) demonstrates that the two cells on the left are endothelial cells and the two cells on the right are 3T3 cells (*B*, *solid arrowheads*). PECAM-1 protein is localized to the intercellular borders on the two endothelial cells (*A*, *solid arrowhead*) and the two transfected 3T3 cells (*A*, *open arrowhead*). In addition, the protein is localized to the border of one of the endothelial cells and a transfected 3T3 cell (*A*, *solid arrow*), demonstrating that the transfected protein functions in a similar manner to the native endothelial cell protein.

The cDNA sequence of PECAM-1 contains two eukaryotic initiation (AUG) codons in the 5' untranslated region, one beginning at base 95 and the other at nucleotide 142. Being largely devoid to T residues and having a purine at the -3 position, both of these codons are flanked by nucleotides that would permit functional initiation of protein synthesis according to the consensus sequence established by Kozak (1987). Fewer than 10% of vertebrate mRNAs contain multiple upstream initiation codons, but ribosomes are known to initiate exclusively at the 5' most ATG codon when it lies in a favorable context (Kozak, 1987). Interestingly, upstream codons that do lie within a Kozak sequence are almost always followed by a termination codon; such is the case for PECAM-1, as the ATG at base 95 is followed by a TAA stop codon only 16 bases downstream. It is therefore likely that after the termination of translation of this 15-bp minicistron, ribosomes reinitiate at base 142 and translate until the stop codon at base 2355.

Recently, two other groups have reported the molecular cloning of PECAM-1 (Stockinger et al., 1990; Zehndar, L. J., K. Hirai, J. L. McGregor, L. J. Levitt, and L. I. K. Leung. 1990. *Blood (Suppl. 1)*. 76:225a.) and noted several minor discrepancies with our earlier published amino acid sequence for this protein (Newman et al., 1990). The variances noted by each of these groups were at different nucleotides, suggesting that PECAM-1 may have several polymorphic forms within the human gene pool. Whether these potentially allelic forms alter the function or specificity of PECAM-1 mediated interactions is not yet known.

Transfection of COS, 3T3 or L cells with a eukaryotic expression vector containing PECAM-1 cDNA resulted in the production and surface expression of a 130-kD glycoprotein (Fig. 3 *A*). The ability of this protein to cross-react with anti-PECAM-1 antibodies and the anti-CD31 mAb confirms the

identity of the expressed protein as PECAM-1. In addition, the reactivity of the transfected protein with the antibodies against endoCAM, a similar molecule to PECAM-1 expressed in bovine cells, confirms that indeed, these two proteins are homologous (Fig. 3 *B*).

The experiments described in this report provide further evidence that PECAM-1 functions as a cell-cell adhesion protein. Analysis of the cellular distribution of the transfected protein clearly shows that PECAM-1 is concentrated at the periphery of adjacent transfected COS and 3T3 cells in a manner reminiscent of its distribution in endothelial cells (Fig. 4). When transfected cells contact nontransfected cells, no cell-cell localization of PECAM-1 occurs. The concentration of PECAM-1 at intercellular junctions of transfected cells and endothelial cells when they are co-cultured suggests that the protein in transfected cells can respond to its natural ligand on adjacent endothelial cells. More direct evidence that PECAM-1 functions as a cell-cell adhesion protein is provided in experiments showing that the molecule supports calcium-dependent cell-cell aggregation when expressed in mouse L cells (Fig. 6) and that this aggregation can be inhibited by an anti-PECAM-1 antibody (Fig. 8). A similar assay has been used to demonstrate the ability of a number of molecules, including N-CAM (Cunningham et al., 1987), cadherins (Takeichi, 1988; Nose et al., 1988), and CD44 (St. John et al., 1989) to participate in cell-cell adhesion.

Although the mechanism(s) whereby PECAM-1 participates in cell-cell adhesion is not yet clear, there are several possibilities, the most obvious being homophilic binding in which PECAM-1 on one cell binds to another molecule of PECAM-1 on an adjacent cell. This possibility is suggested by the finding that PECAM-1 localizes to cell-cell borders only when a fibroblast transfected with PECAM-1 is in con-

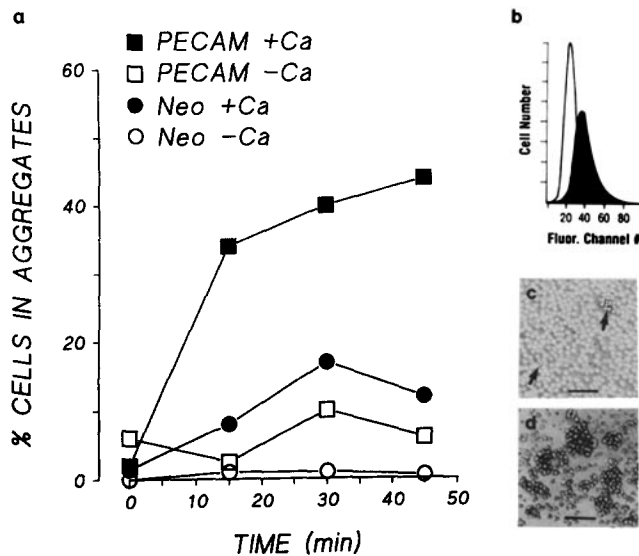


Figure 6. PECAM-1 mediates the calcium-dependent aggregation of transfected L cells. L cells transfected with PECAM-1 cDNA (*PECAM*) or with the neomycin resistance gene only (*Neo*) were nonenzymatically resuspended from tissue culture dishes, washed, and incubated at 37°C under gentle agitation in the presence (+Ca) or absence (-Ca) of 1 mM Ca²⁺ as described in Materials and Methods. (a) The percentage of cells in aggregates was determined as a function of time. The data from one representative experiment is shown. Standard deviations are not indicated in the figure, but were always < 5–10%. (b) Expression of PECAM-1 by transfectants used for this figure was assessed by fluorescence activated cell sorting using mAb hec7. Open curve represents staining profile of control (*Neo*) transfectants; shaded curve represents profile of PECAM-1 transfectants. 65% of the transfected L cells showed levels of fluorescence greater than the control cells. Human umbilical vein endothelial cells show a peak fluorescence of 45 on this scale. (c) Appearance of L cells transfected with neomycin resistance gene after 30 min in the presence of Ca²⁺. Arrows point to occasional aggregates that are formed by control cells. (d) Appearance of L cells transfected with PECAM-1 cDNA after 30 min in the presence of Ca²⁺. Extensive aggregation is seen. Bars, 100 μm.

tact with another transfected cell (Fig. 4) or in contact with a cell that normally expresses PECAM-1 on its surface such as an endothelial cell (Fig. 5). In addition, the expression of PECAM-1 in normally nonaggregating L cells promotes their self aggregation. The calcium dependence of this process (Fig. 6), however, differs from the well-documented ability of members of the immunoglobulin superfamily to mediate homophilic aggregation in the absence of calcium. For example, calcium-independent aggregation has been demonstrated for N-CAM, myelin Po protein, L1, Fasciclin 1, Thy 1, CEA, and myelin-associated glycoprotein (Edelman, 1988; Filbin et al., 1990; Rathjen and Schachner, 1984; Elkins et al., 1990; He et al., 1991; Benchimol et al., 1989; Poltorak et al., 1987).

In addition to its presence at the intercellular junctions of cells that grow in monolayers (i.e., endothelial cell and transfected fibroblasts), PECAM-1 is also expressed on the surface of cells that normally exist in suspension, including peripheral blood monocytes, neutrophils, platelets, and certain T cell subsets. In this light, it is notable that PECAM-1 mediated aggregation of transfected L cells, which also grow

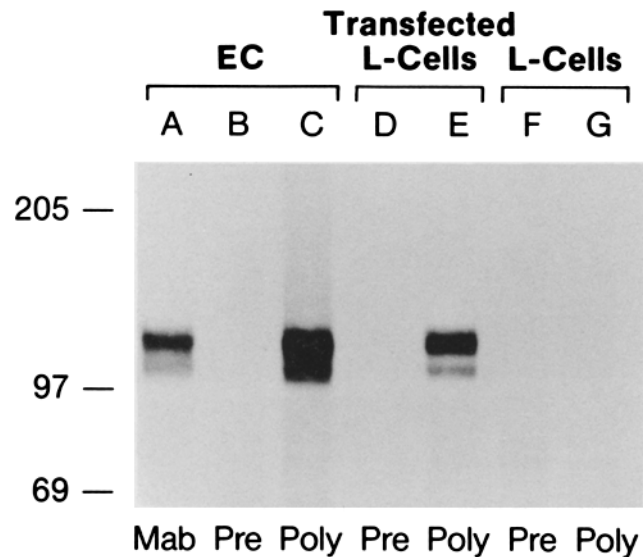


Figure 7. Polyclonal anti-PECAM-1 antibody specifically recognizes PECAM-1 in human umbilical vein endothelial cells and L cells transfected with PECAM-1. Human umbilical vein endothelial cells (A–C), L cells transfected with PECAM-1 (D and E), and nontransfected L cells (F and G) were labeled metabolically with [³⁵S]methionine. Nonionic detergent extracts were then immunoprecipitated with anti-PECAM mAb (A), a 1:250 dilution of pre-immune rabbit serum (B, D, and F) or a 1:250 dilution of anti-PECAM-1 polyclonal antibody (C, E, and G). Both the mAb and the polyclonal antiserum identified the characteristic doublet at 130–120 kD in human endothelial cells. Preimmune serum was nonreactive. In addition, the polyclonal anti-PECAM antiserum identified PECAM protein in the transfected L cells used in the aggregation assay but not in nontransfected L cells. No proteins were detected in either cell line with preimmune serum. Molecular weight markers in kilodaltons are shown on the left.

in suspension, was found to be calcium dependent (Fig. 6). This raises the interesting possibility that PECAM-1-mediated cellular interactions may be heterophilic in certain cell types, that is, PECAM-1 on one cell binds to a different molecule on the cell it contacts. Consistent with this idea, a number of calcium dependent heterophilic interactions mediated by members of the immunoglobulin superfamily have been described. These include ICAM-1 and ICAM-2 binding to β₂ integrins (Staunton et al., 1990), and VCAM-1 binding to VLA-4, a β₁ integrin (Elices et al., 1990). Whether this reflects the requirement for divalent cations of only the integrin involved or also the immunoglobulin superfamily counter-receptor is not known. Recently, a mouse carcinoembryonic antigen gene family member (mmCDM2), also a member of the immunoglobulin superfamily, was shown to require calcium to permit aggregation (Turbide et al., 1991). However, it was not determined if this represented a homophilic or heterophilic interaction.

If PECAM-1 participates in heterophilic binding, the question arises as to the identity of the counter receptor. One possibility, as discussed above, is that it could be a member of the integrin family. The presence of a consensus glycosaminoglycan recognition sequence in its extracellular domain also raises the possibility that PECAM-1 binds to a proteoglycan expressed on an adjacent cell. This sequence, X-B-B-X-B-X, where B is a basic residue and X is a hydrophobic res-

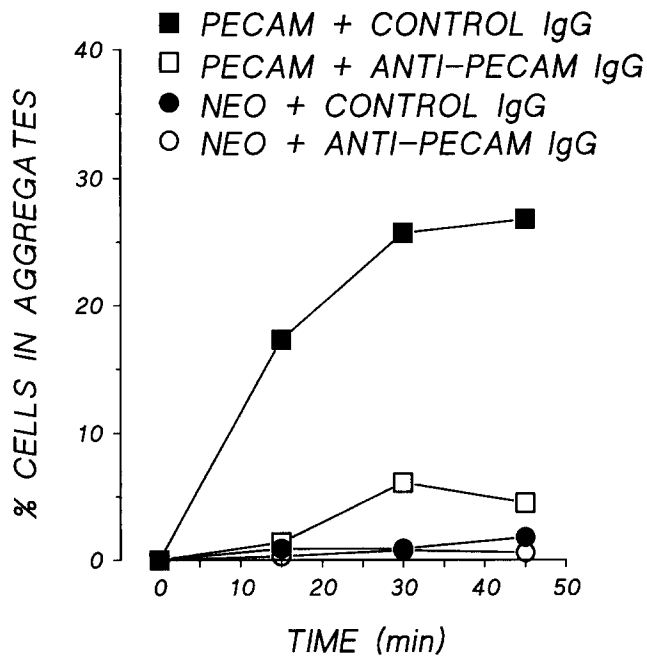


Figure 8. Aggregation of PECAM-1 transfectants is selectively inhibited by anti-PECAM-1 antibody. L cells transfected with PECAM-1 cDNA (PECAM) or with neomycin resistance gene only (NEO) were nonenzymatically resuspended from tissue culture dishes, washed, incubated in 25 μ g/ml rabbit anti-PECAM-1 IgG (ANTI-PECAM IgG) or the same concentration of nonimmune rabbit IgG (CONTROL IgG) for 20 min at 4°C, washed again, and incubated at 37°C under gentle agitation in the presence of 1 mM Ca^{2+} , as described in Materials and Methods. Data points were taken as for Fig. 6. The data are representative of many experiments. Standard deviations were less than the dimensions of the symbols on the graph.

idue, as described by Cardin and Weintraub (1989), is located in a hydrophilic segment at the transition between proposed β -pleated sheet D and E in loop 2 of PECAM-1 (Newman et al., 1990) at amino acids 151–156 (LKREKN). A similar glycosaminoglycan recognition sequence is located in almost the same place in loop 2 of the N-CAM molecule (Reyes et al., 1990). In this case, N-CAM has been shown to interact with heparan sulfate glycosaminoglycan (Cole et al., 1986). Thus, it is possible that PECAM-1 could mediate heterophilic adhesive events either via proteoglycan or integrin counter receptors.

The involvement of a second, different molecule such as a proteoglycan does not necessarily indicate heterophilic adhesion. It may be that the second molecule is required as a cofactor. This mechanism has been evoked to explain the heparan sulfate proteoglycan dependence of N-CAM (Cole and Glaser, 1986; Cole et al., 1986; Reyes et al., 1990) and L1 (Kadmon et al., 1990) mediated neural cell adhesion. This mechanism, or a strictly heterophilic mechanism, could function in the case of PECAM-1 mediated L cell aggregation given the ubiquitous distribution on proteoglycans on cell surfaces.

The mechanisms whereby an immunoglobulin superfamily molecule may promote intercellular adhesion are both many and complex. At least one member of this superfamily, Neural-glial CAM (Ng-CAM) can act as both a homophilic and heterophilic receptor depending upon the type of cell

with which it interacts (Grumet and Edelman, 1988). Given that PECAM-1 is expressed on the surface of a wide variety of cells, some of which are organized into tissues, such as endothelial cells, and others of which interact with different cells only under specific conditions of inflammation or blood clotting, it is possible that a different mechanism may be involved depending upon the specific adhesive event. Regardless of the mechanism by which PECAM-1 promotes adhesion, its presence in relatively large quantities on platelets, leukocytes, and endothelial cells suggests that it may be important in a wide variety of vascular processes.

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