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Brief Definitive Report

A Heterophilic Adhesion Mechanism for Platelet/Endothelial Cell Adhesion Molecule 1 (CD31)

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Summary

The molecular nature of cell adhesion mediated by platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31) was examined using stably transfected L cells in a PECAM-dependent aggregation assay. This adhesion was temperature sensitive and divalent cation dependent, with Mg^{2+} supporting aggregation to a greater degree than Ca^{2+}. PECAM-dependent aggregation was heterophilic: PECAM-1 transfectants bound as readily to control-transfected L cells as to other PECAM-1 transfectants, demonstrating that a molecule endogenously expressed on the L cells serves as the ligand for PECAM in this system and presumably substitutes for the natural human ligand.

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Platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31) is a member of the Ig gene superfamily of cell adhesion molecules (CAMs) (1). It is an integral membrane glycoprotein expressed by all continuous human endothelia in situ (2) and by platelets and cells of myeloid lineage (3–5). Previous studies have suggested that PECAM-1 may function in intercellular recognition and/or adhesion between endothelial cells (EC) since: (*a*) it was expressed diffusely on subconfluent EC but concentrated in the intercellular junctions when cells contacted each other (2); (*b*) antibodies against PECAM could delay the formation of confluent EC monolayers (6; and Muller, unpublished observations); and (*c*) L cells transfected with PECAM cDNA aggregated in a PECAM-dependent fashion that was inhibitable with anti-PECAM antibody (7).

COS cells transiently transfected with PECAM-1 cDNA concentrated the molecule at the junctions between cells expressing the molecule, but not at junctions between transfected and nontransfected cells nor at the free cell borders (7). This strongly suggested a homophilic adhesion mechanism in which PECAM-1 served as both ligand and receptor. The calcium dependence of PECAM-mediated aggregation (7) is unusual for a member of the Ig supergene family, especially for one mediating homophilic aggregation, although precedence for such a mechanism has been established in a related molecule of the carcinoembryonic antigen family (8).

To further characterize the nature of PECAM-mediated adhesion, we studied aggregation of PECAM-1-transfected L cells, a cellular system in which the adhesion events of aggregation can be studied in isolation from other adhesion events involved in binding to substratum or extracellular matrix.

Materials and Methods

Cell Culture. L cells stably transfected with PECAM-1 cDNA were cultured in DME supplemented with 10% FCS and 0.5 mg/ml G418 (Gibco Laboratories, Grand Island, NY). Stable L cell transfectants expressing L-CAM were the generous gift of Dr. Kathryn Crossin (The Rockefeller University).

PECAM-1 Transfectants. The PECAM-1-transfected L cell lines A and SA, as well as the control transfectant line (Neo) bearing neomycin resistance only, have been previously described (7). Line SA cells were derived from line A by FACS® (Becton Dickinson & Co., Mountain View, CA) of high PECAM expressors. In these lines, PECAM and neomycin resistance were cotransfected on separate plasmids.

Lines B1 and D6 were made with PECAM-1 and neomycin resistance on the same plasmid. PECAM was subcloned into pcDNAI/Neo (Invitrogen, San Diego, CA) at the HindIII site by excising PECAM cDNA from the original pGEM7 vector (1) and ligating on synthetic HindIII sites. Unique BamHI sites in both PECAM and the vector allowed unambiguous determination of the orientation of the PECAM insert. Stable L cell transfectants were made by electoporation of L cells (0.5 ml at 2×10^7/ml in DME) with 20 μg linearized plasmid in a gene pulser (Bio-Rad Laboratories, Richmond, CA) at 250 mV, 960 μF, 4-mm path length cuvettes. After 2 d in nonselective medium (DME + 10% FCS), transfectants were selected by addition of the neomycin analogue G418 to a final concentration of 0.5 mg/ml. Neomycin-resistant colonies were picked 10–14 d later, expanded, and tested for PECAM expression by immunofluorescence microscopy using mAb hec7 (2). Line B1 contains the PECAM cDNA in the sense orientation; line D6 contains PECAM in the antisense orientation and is used as a negative control.

Aggregation of L Cell Transfectants. The aggregation assay was performed and quantitated as previously described (7). In certain

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experiments, cells were prelabeled with 5-(and 6-)carboxyfluorescein diacetate succinimide ester (CFSE; Molecular Probes, Eugene, OR) (9). Transfectants in 80-mm culture dishes were washed three times in HBSS, then incubated at 37°C for 10 min in 10 ml of 33 μM CFSE in HBSS (diluted from 10 mM stock in DMSO). After incubation, cells were washed twice in HBSS, then resuspended and processed as described (7).

In experiments to determine whether aggregation was heterophilic or homophilic, two populations of cells, one labeled and the other unlabeled, were resuspended at 2 × 10^6 cells/ml, and 0.5-ml aliquots combined in the wells of a 24-well tissue culture tray. After the aggregation assay was complete, the cells were viewed and photographed under UV light with fluorescein filters using a Nikon Microphot equipped with a UFX-II camera system. Quantitative analysis of the aggregating cell populations was performed as described (10).

FACS® Analysis. L cell transfectants were nonenzymatically resuspended in 10 mM EDTA/HBSS, washed twice in cold HBSS, and resuspended in to a final concentration of 2 × 10^6/ml in 200 μl HBSS containing 10 μl hec7 anti-PECAM mAb culture supernate (2) or isotype-matched mAb as a negative control (final concentration of mAb, ~3 μg/ml). Cells were incubated in 96-well round-bottomed culture trays (Corning, Corning, NY) at 4°C for 30 min, washed three times in HBSS by centrifugation, and resuspended in fluoresceinated $F(ab')_2$ fragments of rabbit anti-mouse IgG (Dako, Santa Barbara, CA) diluted 1:50 in HBSS. The incubation and washing steps were repeated, and the washed cells were analyzed on a FACScan® using Consort 30 software.

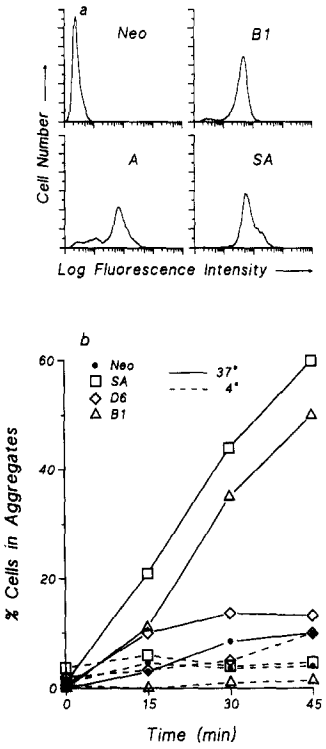

Figure 1. (*a*) Quantitation of PECAM-1 surface expression by independently derived L cell transfectants. PECAM transfectant lines B1, A, and SA, and control transfectant line Neo were nonenzymatically resuspended and analyzed for surface expression of PECAM. Fluorescence intensity histograms reveal reproducible profies with PECAM expression by B1 < A < SA. (*b*) PECAM-1-mediated aggregation is temperature sensitive. The aggregation assay was carried out on resuspended PECAM transfectants (B1, SA) or matching control transfectants (D6, Neo) at 37°C (*solid lines*) or 4°C (*broken lines*). Whereas transfectants expressing PECAM aggregate readily at 37°C, only background levels of aggregation are displayed by the controls. No aggregation of any cell line is seen at 4°C. SD of measurements was <5%.

Results and Discussion

Several different lines of PECAM-1 transfectants were used in these studies; all express PECAM-1 within the physiologic range. Control transfectants (Neo) showed no surface PECAM detectable by FACScan® (Fig. 1 *a*), while the PECAM-1 transfectants displayed their characteristic and reproducible fluorescence profiles with PECAM staining intensity of B1 < A < SA. Under the staining conditions used here, human umbilical vein endothelial cells from confluent cultures have a mean fluorescence channel number of ~100 (data not shown).

Cation Dependence of PECAM-1 Aggregation. We previously reported that PECAM-dependent aggregation of transfected L cells required physiologic concentrations of calcium (7). Since a divalent cation requirement for adhesion mediated by an Ig superfamily molecule is unusual (the notable exceptions being VCAM and ICAM that have as their ligands β_1 and β_2 integrins, respectively [11, 12]), we set out to further investigate the divalent cation dependence of the aggregation mediated by PECAM-1.

Substitution of magnesium (1 mM) for calcium (1 mM) in the aggregation assay led to a 15–40% greater aggregation of PECAM transfectants by 30 min in four separate experiments. Aggregation in the presence of magnesium, as for calcium (7), was blocked by antibody against PECAM (data not shown). Manganese caused a nonspecific aggregation of cells, including control transfectants, that was not blocked by anti-PECAM antibodies.

Aggregation of PECAM-1 Transfectants Is Temperature Sensitive. Aggregation of PECAM-expressing transfected L cells occurred readily at 37°C, but not at 4°C (Fig. 1 *b*). Control cells did not aggregate significantly at either temperature. The temperature dependence of aggregation is similar to that exhibited by integrins and cadherins (13), and clearly different from the temperature-insensitive nature of binding mediated by selectins (14).

Aggregation Mediated by PECAM-1 Is Heterophilic. To define whether adhesion in this system was homophilic or heterophilic, we performed a mixing experiment similar to that used for other CAMs (13, 15). L cells transfected with the neomycin resistance gene only (Neo) or with PECAM-1 in the antisense orientation (D6) were vitally labeled with the fluorescent dye CFSE and mixed with an equal number of unlabeled PECAM-expressing transfectants in the standard aggregation assay. Aggregates were removed after 30–45 min and examined by fluorescence microscopy. A homophilic adhesion mechanism would produce only aggregates of transfected (unlabeled) cells. On the other hand, a heterophilic-adhesive mechanism, wherein PECAM-1 binds to a different mole-

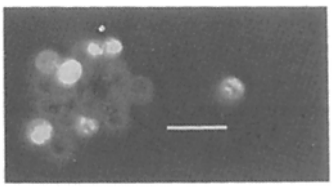

Figure 2. PECAM-1-mediated aggregation is heterophilic. PECAM-transfected L cells were mixed with equal numbers of CFSE-labeled control transfectants in the aggregation assay for 30 min at 37°C. This micrograph is a composite overlay of a typical aggregate viewed under phase contrast and fluorescence optics. Cells expressing PECAM bind to those that do not. Nontransfected cells are frequently seen at the edges of aggregates indicating that they are not nonspecifically trapped in these cell clusters. Identical results are obtained when PECAM transfectants are the cell population bearing the CFSE label. Bar = 100 μm.

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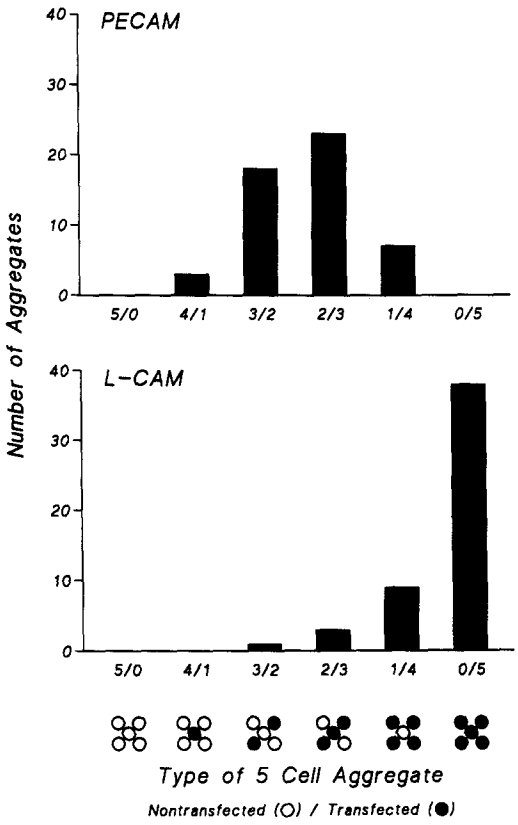

Figure 3. Quantification of heterophilic aggregation. Control transfected cells (line D6) were labeled with CFSE and mixed with equal numbers of PECAM transfectants (line B1) or L-CAM transfectants in an experiment similar to the one performed for Fig. 2. Aggregates at 45 min were examined by fluorescence microscopy. This figure compares randomly chosen aggregates of five cells observed in both the PECAM and L-CAM samples. The histograms show the number of aggregates bearing increasing numbers of the specific transfected cell type. The L-CAM sample distribution is skewed far to the right, as expected for a homophilic aggregation, whereas the PECAM sample shows a unimodal peak just to the right of center, as expected for a heterophilic aggregation mechanism (10) in which PECAM transfectants can bind equally well to transfected or nontransfected cells. Skewing just to the right of the midline stems from the higher probability of nucleating an aggregate with a transfected (PECAM-1-expressing) cell. In nonmathematical terms, if the first cell in the nascent aggregate expresses PECAM, then the next cell to join the aggregate may be transfected or not if binding is heterophilic. However, if the first cell is nontransfected, the next cell must be transfected (PECAM expressing) in order to bind and continue the growth of the aggregate.

cule on the apposing cell, would produce mixed aggregates of transfected (unlabeled) and nontransfected (labeled) cells.

The results of such an experiment (Fig. 2) showed that aggregation was clearly heterophilic, with nontransfected cells positively identified in aggregates by virtue of their bright fluorescence. Anti-PECAM antibody markedly inhibited aggregation in this system, as previously shown (7).

The heterophilic nature of this aggregation was consistently observed in all three lines of PECAM transfectants. However, to control for our ability to detect a homophilic adhesion mechanism if one were occurring, we compared in parallel the aggregation of L cells transfected with the liver cell adhesion molecule L-CAM (the chicken equivalent of E-cadherin) (16) and those transfected with PECAM. L-CAM mediates calcium-dependent homophilic adhesion (17). L-CAM-expressing cells aggregated in a clearly homophilic manner, with >80% of the aggregates containing only L-CAM transfectants, and the majority of the rest containing only one nontransfected cell. In contrast, the PECAM transfectants formed mixed aggregates with controls, as previously observed. Fig. 3 shows the results for aggregates of five cells in this experiment, but is typical of the results for all sizes examined (3 to >20 cells).

The characteristics of PECAM-mediated adhesion described in this report are intrinsic features of the adhesion molecule, since transfected cells derived from different parental L cell lines using different vectors behaved identically in these experiments. The ligand for PECAM in this system must be a molecule(s) for which endogenous surface components of (murine) L cells can substitute. This opens up the possibility that cells not bearing PECAM can interact with PECAM on endothelium or leukocytes in vivo. The temperature and divalent cation dependence, and the precedent set by the other vascular CAMs of the Ig superfamily, ICAM-1 and VCAM-1, suggest that the ligand for PECAM-1 could be an integrin. On the other hand, the second Ig loop of PECAM contains a consensus glycosaminoglycan recognition sequence (LKREKN) (1, 5, 7, 18), suggesting that PECAM, like neural cell adhesion molecule (N-CAM), which has a similar sequence at the same site (19), could bind a glycosaminoglycan moiety (20).

Identification of a heterophilic adhesion mechanism for PECAM-1 was somewhat surprising in view of our results showing that PECAM was localized exclusively at borders between PECAM-transfected COS cells (7), a finding that suggested homophilic adhesion. However, this does not rule out the possibility that PECAM-1 could mediate homophilic adhesion under different conditions. Dual homophilic/heterophilic adhesion has been demonstrated for neuron-glia cell adhesion molecule (Ng-CAM) (21). The aggregation assay is a short-term reaction in which hydrodynamic forces tend to push the suspended cells together. In contrast, cells in culture have hours to days in which molecules on apposing membranes may reorganize to create the most stable adhesion. It is possible, for example, that the initial contact of endothelial cells with each other involves heterophilic adhesion via PECAM-1, which sorts out into homophilic adhesion as the cells become more closely apposed.

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