Regulation of PECAM-1 in Endothelial Cells during Cell Growth and Migration

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Endothelial cells (EC) that form the inner lining of blood vessels remain quiescent in the normal adult vasculature except during angiogenesis and reendothelialization, which result in EC proliferation and migration. EC placed in culture at subconfluent density also undergo cell multiplication and movement. This report demonstrates that whereas in confluent EC in a compact monolayer, the EC-EC adhesion molecule platelet-endothelial cell adhesion molecule-1 (PECAM-1) is strongly expressed at cell borders, little or no PECAM-1 immunostaining is detected in sparse or migrating cultured EC. Consistent with this observation, steady-state PECAM-1 mRNA expression was much lower in subconfluent EC than in confluent EC. The absence of PECAM-1 expression in sparse EC appeared not to be linked to ability to proliferate, since PECAM-1 expression remained low even in the presence of nitric oxide (NO) or mitomycin C, agents that inhibit EC growth. However, another growth-inhibitory agent, TGF-β1, did not alter PECAM-1 staining. Based on these observations, it is hypothesized that cell-associated mechanical forces underlying cell tensegrity regulate PECAM-1 expression.

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In normal adults, endothelial cells (EC) remain in a contact-inhibited confluent monolayer that allows for little room for proliferation of movement of cells. During angiogenesis (the growth of new blood capillaries from preexisting vessels) and reendothelialization, repair of denuded endothelium (following balloon angioplasty, bypass grafting, radiation, or other injurious processes), pronounced proliferation and migration of EC occur. In addition, EC placed into culture retain the ability to multiply and migrate until they form a confluent monolayer. Responses of cultured EC to physiologic effectors (e.g., angiogenic agents) are generally similar to those observed *in vivo*. Consequently, several models of angiogenesis and reendothelialization have been developed *in vitro* to deduce mechanisms underlying the regulation of EC growth and movement.

Pronounced changes in cell-cell contact occur both during cell proliferation and cell migration. As cell density increases, so does cell-cell adhesion, ultimately leading to contact inhibition of growth. Cell migration requires migrating cells to sever connections with neighboring cells. Because of the association between cell-cell adhesion and cell growth and migration, we studied the expression and localization of the EC-EC adhesion molecule platelet-endothelial cell adhesion molecule-1 (PECAM-1; also called CD31), which is associated with the regulation of cell migration and angiogenesis (1–6), in sparse and confluent cultured EC, as well as in migrating EC.

In an *in vitro* model of reendothelialization (7), an injury is made in the middle of a confluent EC monolayer, inducing EC from intact areas of the monolayer to migrate into the denuded region, proliferate, and eventually repair the injury. These migrating cells sever connections with cells of the intact area of the monolayer, remain solitary for sometime, undergo morphologic changes, and form new cell-cell contacts. Our results indicate that profound changes in PECAM-1 expression takes place in EC at different stages of growth and movement. These changes occur at the protein and the steady-state mRNA level. The alterations in PECAM-1 expression seem unrelated to the proliferative ability of EC. However, the fact that cell spreading and cell-matrix interactions correlate with PECAM-1 expression prompts us to hypothesize that structural integrity of the cell, termed cellular tensegrity (8, 9), regulates PECAM-1 expression. Because PECAM-1 is involved not only in EC-EC adhesion, but also in EC-leukocyte binding, our findings may be significant in deciphering the molecular mechanisms underlying leukocyte transmigration during inflammation.

Materials and Methods

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Seattle, WA) and were grown in EBM media supplemented with 10%

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fetal calf serum (FCS) and bovine brain extract (Clonetics). Bovine pulmonary arterial endothelial cells (BPAEC) were purchased from ATCC (Rockville, MD) and were grown in RPMI medium supplemented with 10% FCS, L-glutamine, and the antibiotics penicillin and streptomycin. The choice of BPAEC for the majority of experiments was dictated by two considerations: BPAEC required a simpler media for growth, and therefore, yielded results less complicated by added growth factors; and BPAEC could be cultured to higher passage numbers without appreciable loss of phenotype or growth rate, thus yielding more cells for experiments (e.g., for RNA isolation).

Generation of PECAM-1 Antibody. Monoclonal antibody to human PECAM-1 was generated as recently described (10). Total RNA was isolated from low passage HUVEC and was used to prepare a full-length human PECAM-1 cDNA. This cDNA was used as a template in PCR amplifications to assemble a PECAM domain 1 and 2-immunoglobulin fusion protein. A PECAM DNA fragment was ligated into human immunoglobulin γ4 constant region-containing expression vector such that the first two PECAM immunoglobulin-like domains were joined to an immunoglobulin γ4 heavy chain in which the VH and CH1 domains had been deleted (1-2PECAM-IgG4). This construct was expressed in SP2/0 murine myeloma cells and was purified to homogeneity from culture supernatants by protein A chromatography. Mice were immunized and boosted with 1-2PECAM-IgG4 fusion protein. Spleen cells were isolated and fused with murine myeloma F/0 cells (ATCC). Clones producing anti-PECAM antibodies were selected by ELISA for their reactivity with immobilized 1-2PECAM-IgG4 and their lack of reactivity with a control IgG4 fusion protein. These clones were subcloned and single clones were isolated and purified antibodies were characterized. An antibody recognizing both human and bovine PECAM-1 was used for these studies.

Other Anti-PECAM-1 Antibodies. In addition to the antibody generated by us, two commercially available monoclonal anti-human PECAM antibodies were used—one from Sigma (St. Louis) and one from Chemicon (Temecula, CA). Neither of them recognized bovine PECAM-1, so these were only used with HUVEC.

***In Vitro* Reendothelialization Model.** The *in vitro* reendothelialization model was done essentially by the method of Herman (7). EC were grown to confluence on two-chamber slides (2.1 cm^2 growth area), and a 2-mm-wide strip of cells in the middle of the monolayer was removed by scraping with a pipet tip. Since measurement of cell migration rate was not an objective of this study, no attempt was made to keep the strip width precisely equal in each experiment. The culture was washed to remove floating cells and was returned to the incubator. Cells in the intact areas of the monolayer migrated into the denuded area from both sides and completely repaired the injury in 2 to 3 days. In general, immunostaining was performed after 24 hr of migration to determine PECAM-1 expression in migrating cells.

Immunocytochemistry. Cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, and washed again with PBS. They were then treated with 0.2% Triton X-100 for permeabilization, washed again, and incubated with anti-PECAM antibody (10 μg/ml in 3% BSA-containing PBS) for 45 min at 37°C. (Permeabilization was performed to detect possible cytosolic pools of PECAM-1). Cells were then washed with PBS three times and then incubated with a FITC-labeled goat anti-mouse IgG (ICN; diluted 1:75 in 3% BSA-containing PBS) for 45 min at 37°C. After washing three times with PBS, cells were coverslipped with antifade mounting media (90% glycerin, 0.1% p-phenylenediamine, pH 9) and viewed under an Optiphot microscope.

Northern Blotting. Total RNA was prepared from confluent (>100,000 cells/cm^2) and sparse (30,000–50,000 cells/cm^2) EC 48 hr after plating using RNAStat (Tel-Test, Friendsville, TX) and following the manufacturer's protocol. RNA was resolved on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled bovine PECAM-1 cRNA probe prepared using a Riboprobe kit (Promega, Madison, WI) at 56° to 60°C overnight. The blot was then washed twice for 15 min in 0.1× SSC, 0.1× SDS at 68°C, and exposed 4 hr with an X-ray film with two intensifying screens.

Cell Proliferation Assay. Cells were plated on 2.1-cm^2 wells of 24-well dishes subconfluent density (about 25,000–30,000 cells/cm^2). The next day, unattached cells were washed off with PBS and the cell number was determined from one well (T_0). Then the test substances (mitomycin C, TGFβ, and SNAP) were added individually in triplicate wells per substance in regular media. After 3 days, cells were washed with PBS, trypsinized, and counted electronically in a Coulter Counter. Graphs were generated by Microsoft Excel and statistics were done with InStat (GraphPad, San Diego, CA).

Reagents and Chemicals. The bovine PECAM-1 cDNA was a generous gift from Dr. P.A. Marsden (Toronto, Canada). All chemicals were purchased from Sigma.

Results

Immunostaining for PECAM-1 in Confluent, Sparse, and Migrating EC. PECAM-1 was ubiquitously expressed at cell-cell borders in confluent (>100,000 cells/cm^2) BPAEC and HUVEC (Fig. 1, A and D). In contrast, staining for pericellular PECAM-1 was nonexistent in sparse (~25,000 cells/cm^2) BPAEC, except areas of cell-cell contact (Fig. 1B). This is consistent with published results (1, 2). Even as cell density increased and more cell-cell contacts were established, full-fledged expression of PECAM-1 did not appear until confluent EC density was reached. Identical results were obtained with HUVEC (not shown).

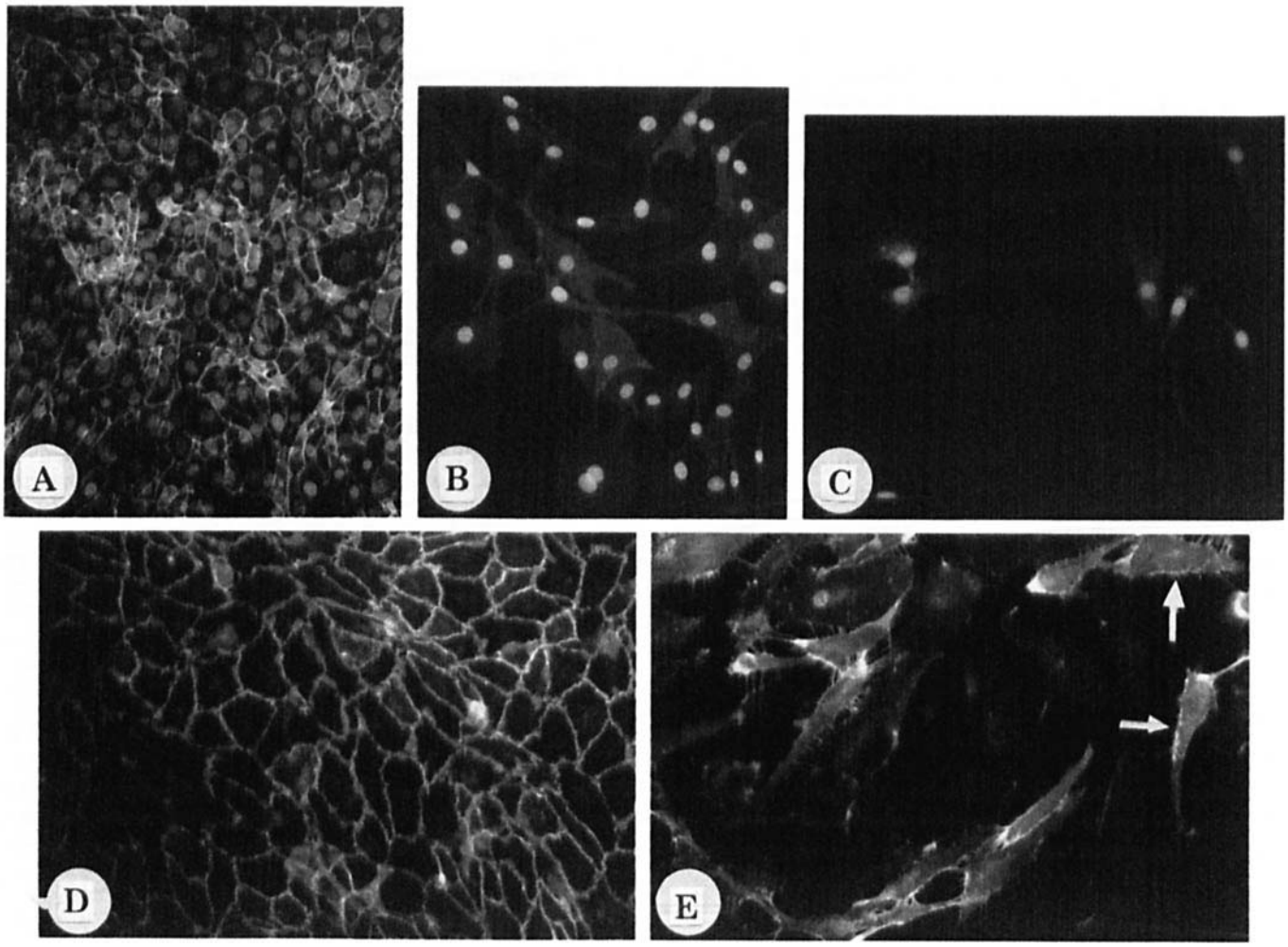

Figure 1. Expression of PECAM-1 at cell-cell borders in BPAEC and HUVEC. Immunostaining of (A) confluent, (B) sparse, and (C) migrating BPAEC with anti-PECAM-1 antibody shows that PECAM-1 is present at almost all cell-cell borders in confluent, but not in sparse or migrating BPAEC. The apparent nuclear staining is an artifact and appeared in low-staining cells even when only the secondary antibody (without primary anti-PECAM antibody) was used as negative control. It is probably due to permeabilization. Immunostaining of (D) confluent monolayer and (E) migrating edge (after denudation) of HUVEC shows that whereas all cells in the intact monolayer express PECAM-1 at cell-cell borders, only a few cells at the migrating edge do. Arrows point to some of the cells that express PECAM-1 at border areas without apparent cell-cell contact. Migrating cells appear larger than cells in the monolayer due to increased spreading.

To determine whether PECAM-1 expression is altered in cells that were part of a confluent monolayer but become isolated (noncontacting) as a result of migration, we performed PECAM-1 immunostaining in the *in vitro* reendothelialization model, as described in "Materials and Methods." As shown in Figure 1C, cells that had migrated to the denuded area after scrape injury showed little or no PECAM-1 immunostaining at cell-cell borders, whereas cells in the intact area of the monolayer expressed high levels similar to confluent monolayers.

Upon closer examination, it was seen that cell-cell contact was not an absolute determinant of PECAM-1 expression. At the injured edge of the monolayer where cells that have just separated from the intact area, most cells are making partial contact or no contact with other cells. Immunostaining for PECAM-1 showed that whereas a subpopulation of migrating cells lacked any PECAM-1 immunoreactivity even though they had some residual cell-cell contacts, other cells expressed PECAM-1 even at border areas where no cell-cell contact occurred (Fig. 1E, some indicated by arrows). Thus, cell-cell contact alone was not the determinant of PECAM-1 expression. The pattern of PECAM-1 immunostaining in HUVEC by our antibody was totally identical to that generated by two other commercially available antibodies as described in "Materials and Methods," indicating these results were not due to any abnormalities in the antibody.

PECAM-1 Expression Is Not Dependent on Proliferative Ability of EC. In addition to migrating ability and degree of cell-cell contact, another difference between confluent EC and sparse or migrating EC is the ability to proliferate. Whereas confluent cells are prevented from proliferation by contact inhibition, sparse and migrating cells proliferate. To determine whether PECAM-1 expression is dependent the proliferative ability of EC, BPAEC were treated with TGFβ1 (2 ng/ml, 72 hr), a well-characterized EC growth inhibitor, in another experiment, they were treated with SNAP (200 μM, 72 hr), a nitric oxide (NO)

donor that inhibits BPAEC growth (11), and in another experiment, they were treated with mitomycin C (25 μg/ml for 30 min), which inhibits cell growth in general by cross-linking DNA. Cells were incubated for an additional 72 hr. Parallel proliferation assays were conducted to ensure these agents were indeed inhibiting BPAEC growth (Fig. 2A). Immunostaining with antiPECAM-1 antibody revealed that there was very low staining in SNAP- or mitomycin-treated growth-arrested BPAEC at cell-cell borders (Fig 2B, 1 and 2) compared with growth-arrested untreated confluent cells, as shown in Figure 1, A and D. This indicated that abrogation of proliferative ability was not inducing PECAM-1 expression. Some punctate staining was observed in both cases in the cytosol, suggesting low amounts of PECAM-1 protein is synthesized, but not transported to the cell membrane. However, TGFβ1-treated EC had higher staining than SNAP- or mitomycin C-treated cells and in some areas comparable with confluent cell staining (Fig. 2). This observation will be commented on in the "Discussion" section.

Steady-State PECAM-1 mRNA Is Expressed at Significantly Higher Levels in Confluent EC than in Sparse EC. To determine whether the increased expression of PECAM-1 protein in confluent EC was at the protein level only or even at the steady-state mRNA level, Northern hybridization was performed with total RNA from confluent and sparse BPAEC RNA against bovine PECAM-1 cRNA probe. As shown in Figure 3, significantly higher levels of PECAM-1 mRNA are present in confluent BPAEC than in sparse BPAEC. The position of the PECAM-1 transcript by approximate measurement corresponded with the 3.7-kb size of bovine PECAM-1 mRNA as reported by Stewart *et al.* (12).

Discussion

Sparse and migrating EC make significantly fewer cell-cell contacts than cells in a confluent monolayer. In this study we report that this reduced cell-cell contact is attended by a reduction in the expression of the cell adhesion molecule PECAM-1, at the protein and steady-state mRNA levels. Thus downregulation of this adhesion molecule may facilitate the release of cells from the intact, quiescent monolayer during reendothelialization. (A recent report [13] claimed PECAM-1 is not involved in maintenance of the EC quiescent monolayer; however, ECV304 cells used by these investigators are now regarded to be of nonendothelial origin [ATCC, personal communication], and publications using that cell line have been excluded from this report).

Several differences between sparse and confluent EC have previously been reported (14–16). These differences suggest that cell-cell contact, as well as proliferative ability of EC, govern many of the biochemical and functional characteristics of EC. Our findings indicate that neither cell-cell contact nor proliferative ability appears to be the sole determinant of PECAM-1 regulation, even though some contribution cannot be ruled out. In addition to cell contact and proliferative ability, however, another difference between

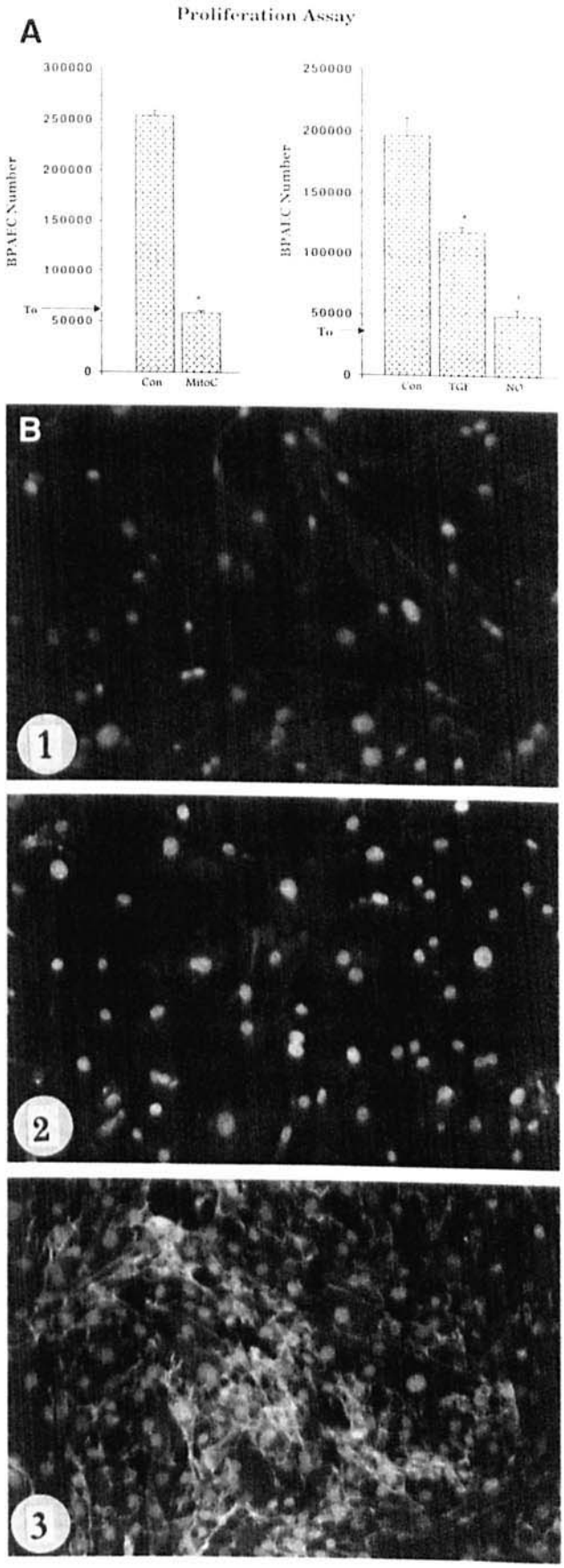

Figure 2. (A) Inhibition of BPAEC proliferation by mitomycin C, TGFβ1, and NO (generated from SNAP). Assays were carried out for 3 days after addition of inhibitors. To, cell number at start of experiment. Asterisk indicates statistically significant reduction compared to control. (B) Immunostaining for PECAM-1 in (1) SNAP-treated, (2) mitomycin-treated, and (3) TGFβ1-treated BPAEC. Magnification is 20×.

sparse or migrating and confluent EC is cell shape and cell spreading—sparse cells are generally more spread out and migrating cells are more elongated than the polygonal cells in a confluent monolayer. The architectural integrity of the cell, termed tensegrity, controls cell shape and spreading and is maintained by mechanical forces associated with the cell such as cell-matrix adhesion, cell-cell adhesion, and the cytoskeleton. Strong evidence suggests changes in cellular tensegrity are mechanochemical regulators of cell function (8, 9). Thus, sparse and migrating EC have a different, more relaxed, tensegrity from confluent EC. In the case of TGFβ1, however, two opposing forces may be regulating PECAM-1 expression; on the one hand, this cytokine inhibits cell growth, thus reducing cell-cell contact forces, but TGF-β1 significantly increases matrix deposition by EC and thereby may strengthen cell-matrix contact forces. This may explain the observation that growth-arrested TGFβ1-treated cells have higher PECAM-1 levels than growth-arrested SNAP- or mitomycin C-treated cells. Taken together, these observations suggest that PECAM-1 expression in EC is at least partially under mechanochemical regulation, with stronger cell-associated mechanical forces favoring PECAM-1 upregulation, and weaker or more relaxed forces favoring suppression. Indeed, an inverse relation has been reported between matrix regulation by TGF-β1 and cell migration (17). Of course, additional studies are required to verify this concept.

Expression of PECAM-1 is altered during angiogenesis (3, 5), a process that requires EC migration. PECAM-1 has been suggested to be required for cell elongation, migration, and/or invasion during angiogenesis (6). Romero *et al.* (18) showed that PECAM-1 downregulation occurs during *in vitro* angiogenesis. Sheibani and Frazier (19) reported that downregulation of PECAM-1 alters EC phenotype, and Dosanjh *et al.* (20) have shown that reduced expression of PECAM-1 occurs in morphologically altered endothelial cells isolated from hemangiomas. Several regulators of angiogenesis such as TNFα and oxygen alter PECAM-1 levels in EC (12, 21). In addition to its adhesive role, PECAM-1 has been reported to have additional regulatory roles through acting as a reservoir for and regulator of tyrosine-phosphorylated β-catenin (22) and inhibition of TCR-mediated signal transduction (23). Thus, the downregulation of PECAM-1 observed by us may be physiologically significant during angiogenesis and reendothelialization. PECAM-1 also mediates EC-leukocyte adhesion during leukocyte extravasation (24), an event critical to inflammation. Blocking antibodies to PECAM-1 has been shown to inhibit acute inflammation *in vivo* (25). Thus, our observations may facilitate understanding of certain steps in the inflammatory process as well, especially in comparing the responses of intact versus denuded endothelium.

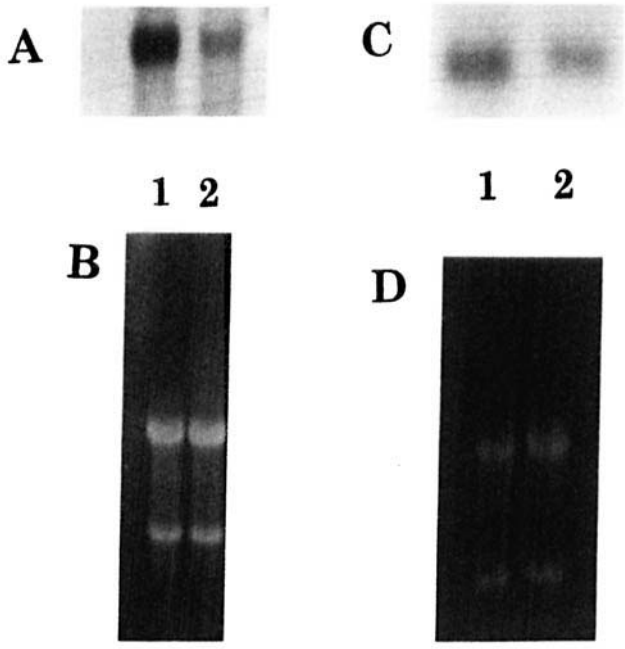

Figure 3. Northern blotting for PECAM-1 in confluent (lane 1) and sparse (lane 2) BPAEC. (A) and (C) show steady-state PECAM-1 mRNA levels; (B) and (D) corresponding ethidium bromide staining shows equal loading in both lanes. (C and D) Enlarged (25%) for better visualization. Confluent cell density was 3 to 4× sparse cells the experiment represented in A and B and 2.5× in experiments C and D.

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1. Albelda S, Oliver P, Romer L, Buck C. EndoCAM: A novel endothelial cell-cell adhesion molecule. J Cell Biol **110:**1227–1237, 1990.
2. Muller W, Ratti C, McDonnell S, Cohn Z. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. J Exp Med **170:**399–414, 1989.
3. Berger R, Albelda SM, Berd D, Ioffreda M, Whitaker D, Murphy GF. Expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1) during melanoma-induced angiogenesis *in vivo*. J Cutan Pathol **20:**399–406, 1993.
4. DeLisser HM, Newman PJ, Albelda SM. Platelet endothelial cell adhesion molecule (CD31). Curr Top Microbiol Immunol **184:**37–45, 1993.
5. DeLisser HM, Christofidou-Solomidou M, Strier RM, Burdick MD, Robinson CS, Wexler RS, Kerr JS, Garland C, Merwin JR, Madri JA, Albelda SM. Involvement of endothelial PECAM-1/CD31 in angiogenesis. Am J Pathol **151:**671–677, 1997.
6. Yang S, Graham J, Kahn JW, Schwartz EA, Gerritsen ME. Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assembly and lumen formation in three-dimensional collagen gels. Am J Pathol **155:**887–895, 1999.
7. Herman IM. Molecular mechanisms regulating the vascular endothelial cell motile response to injury. J Cardiovasc Pharmacol **22:**S25–S36, 1993.
8. Ingber D. Control of capillary growth and differentiation by extracellular matrix: Use of a tensegrity (tensional integrity) mechanism for signal processing. Chest **99:**34S–40S, 1991.
9. Ingber D. Cellular tensegrity: Defining the rules of biological design that govern the cytoskeleton. J Cell Sci **104:**613–627, 1993.
10. Nakada MT, Amin K, Christofidou-Solomidou M, O'Brien CD, Sun J, Gurubhagavatula I, Heavner GA, Taylor AH, Paddock C, Hong-Sun Q, Zehnder J, Newman PJ, Albelda SM, DeLisser HM. Antibodies against the first Ig-like domain of human platelet endothelial cell adhesion molecule-1 (PECAM-1) that inhibit PECAM-1-dependent ho-

mophilic adhesion block in vivo neutrophil recruitment. J Immunol **164**:452–462, 2000.
11. RayChaudhury A, Frischer H, Malik AB. Inhibition of endothelial cell proliferation and bFGF-induced phenotypic modulation by nitric oxide. J Cell Biochem **63**:125–134, 1996.
12. Stewart RJ, Kashour TK, Marsden PA. Vascular endothelial platelet endothelial cell adhesion molecule-1 (PECAM-1) expression is decreased by TNF-α and IFN-γ. J Immunol **156**:1221–1228, 1996.
13. Halama T, Staffler G, Hoch S, Stockinger H, Wolff K, Petzelbauer P. Vascular-endothelial cadherin (CD144) but not PECAM-1- (CD31) based cell-to-cell contacts convey the maintenance of a quiescent endothelial monolayer. Int Arch Allergy Immunol **120**:237–244, 1999.
14. Castellot JJ, Addonizio ML, Rosenberg R, Karnovsky MJ. Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cells. J Cell Biol **90**:372–379, 1981.
15. Dodge A, Lu X, D'Amore PA. Density-dependent endothelial cell production of an inhibitor of smooth muscle cell growth. J Cell Biochem **53**:21–31, 1993.
16. RayChaudhury A. Differential sensitivity of cultured endothelial cells to peroxynitrite and hydrogen peroxide toxicity: *In Vitro* Cell Dev Biol **32A**:593–596, 1996.
17. Basson C, Kocher O, Basson M, Asis A, Madri J. Differential modulation of vascular cell integrin and extracellular matrix expression *in vitro* by TGF-beta1 correlates with reciprocal effects on cell migration. J Cell Physiol **153**:118–128, 1992.
18. Romero L, Zhang D-N, Herron G, Karasek M. Interleukin-1 induces major phenotypic changes in human skin microvascular endothelial cells. J Cell Physiol **173**:84–92, 1997.
19. Sheibani N, Frazier W. Down-regulation of platelet endothelial cell adhesion molecule-1 results in thrombospondin-1 expression and concerted regulation of endothelial cell phenotype. Mol Biol Cell **9**:701–713, 1998.
20. Dosanjh A, Chang J, Bresnick S, Zhou L, Reinisch J, Longaker M, Karasek M. *In vitro* characteristics of neonatal hemangioma endothelial cells: Similarities and differences between normal neonatal and fetal endothelial cells. J Cutan Pathol **27**:441–450, 2000
21. Zhou L, Dosanjh A, Chen H, Karasek M. Divergent effects of extracellular oxygen on the growth, morphology, and function of human skin microvascular endothelial cells. J Cell Physiol **182**:134–140, 2000.
22. Ilan N, Mahooti S, Rimm DL, Madri J. PECAM-1 (CD31) functions as a reservoir for and a modulator of tyrosine-phosphorylated β-catenin. J Cell Sci **112**:3005–3014, 1999.
23. Newton-Nash DK, Newman P. A new role for platelet endothelial cell adhesion molecule-1 (CD31): Inhibition of TCR-mediated signal transduction. J Immunol **163**:682–688, 1999.
24. Muller W, Weigl S, Deng X, Phillips D. PECAM-1 is required for transendothelial migration of leukocytes. J Exp Med **178**:449–460, 1993.
25. Bogen S, Pak J, Garifallou M, Deng X, Muller WA. Monoclonal antibody to murine PECAM-1 (CD31) blocks acute inflammation *in vivo*. J Exp Med **179**:1059–1064, 1994.