Spatial and Temporal Relationships between Cadherins and PECAM-1 in Cell–Cell Junctions of Human Endothelial Cells

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Abstract. The integrity of the endothelial layer, which lines the entire cavity of the vascular system, depends on tight adhesion of the cells to the underlying basement membrane as well as to each other. It has been previously shown that such interactions occur via membrane receptors that determine the specificity, topology, and mechanical properties of the surface adhesion. Cell–cell junctions between endothelial cells, in culture and in situ, involve both Ca^{2+}-dependent and -independent mechanisms that are mediated by distinct adhesion molecules. Ca^{2+}-dependent cell–cell adhesion occurs mostly via members of the cadherin family, which locally anchor the microfilament system to the plasma membrane, in adherens junctions. Ca^{2+}-independent adhesions were reported to mainly involve members of the Ig superfamily.

In this study, we performed three-dimensional microscopic analysis of the relative subcellular distributions of these two endothelial intercellular adhesion systems. We show that cadherins are located at adjacent (usually more apical), yet clearly distinct domains of the lateral plasma membrane, compared to PECAM-1. Moreover, cadherins were first organized in adherens junctions within 2 h after seeding of endothelial cells, forming multiple lateral patches which developed into an extensive belt-like structure over a period of 24 h. PECAM-1 became associated with surface adhesions significantly later and became progressively associated with the cadherin-containing adhesions. Cadherins and PECAM-1 also differed in their detergent extractability, reflecting differences in their mode of association with the cytoskeleton. Moreover, the two adhesion systems could be differentially modulated since short treatment with the Ca^{2+} chelator EGTA, disrupted the cadherin junctions leaving PECAM-1 apparently intact.

These results confirm that endothelial cells possess distinct intercellular contact mechanisms that differ in their spatial and temporal organization as well as in their functional properties.

Cell junctions are specialized membrane domains that provide mechanical intercellular coupling and play a key role in tissue morphogenesis, intercellular communication, and the generation of transcellular forces (Cunningham and Edelman, 1990). Beyond their direct mechanical roles, cell adhesions are also believed to be responsible for a variety of long term dynamic processes including cell locomotion, proliferation, and differentiation (Edelman, 1993; Geiger, 1993; Hedrick et al., 1993).

As may be expected from their diversified functions, cell adhesions are also heterogenous from the molecular point of view, involving a variety of transmembrane molecules, different cytoskeletal networks, and specific anchoring pro-

teins which link the two (Geiger and Ginsberg, 1991; Geiger et al., 1992; Fartasch et al., 1993; Kaiser et al., 1993).

The present study addresses the molecular diversity of cell adhesions in human endothelial cells. Endothelial junctions are known to be involved in a large variety of processes that affect, in a major way, the morphogenesis and physiology of the vessel wall (Franke et al., 1988). The functional importance of these junctions is apparent from the various pathological disorders, which are associated with altered endothelial adhesion, including atherosclerosis, thrombosis, embolism, hypertension, and hemorrhagic disorders (Wu et al., 1988; Jensen et al., 1989; Bar et al., 1991; Frostegard et al., 1991; Rubin, 1992; Arnaout, 1993). Moreover, changes in the endothelium may affect extravasation or intravasation of cells including inflammatory cells and metastases (Aznavoorian et al., 1993; Behrens, 1993; Dorudi and Hart, 1993).

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Electron microscopic studies have revealed various structurally distinct junctional elements in endothelia, including tight junctions, gap junctions, and actin-associated adherens-type junctions (AJ)[1] (Schneeberger and Lynch, 1984; Franke et al., 1988; Milton and Knutson, 1990). In addition, the basal membrane of these cells is tightly attached to the underlying basement membrane (Albelda and Buck, 1990; Ingber, 1991a,b). The intercellular junctions and in particular the AJ, form a continuous belt at the periphery of each cell, similar (though topologically less uniform and sharply defined) to the zonula adherens of polar epithelial cells. The presence of AJ in endothelia, both in culture and in situ, was demonstrated mostly by the specific immunolocalization of the different junctional proteins (Franke et al., 1988; Salomon et al., 1992; Stolz et al., 1992). Actin was shown to form a belt along the junctional membrane, accompanied by typical AJ "plaque" molecules such as vinculin and plakoglobin.

Studies of the surface receptors which mediate endothelial intercellular adhesion focused on three distinct families of adhesion molecules, including cadherins, integrins, and members of the Ig superfamily. The cadherin family consists of a large group of structurally related transmembrane glycoproteins, whose expression is developmentally regulated (Takeichi, 1991; Geiger and Ayalon, 1992). They mediate homophilic, calcium-dependent cell adhesion, and are specifically associated with AJ. Several cadherins were recently identified in bovine and human endothelial cells. In the bovine endothelium, at least two distinct cadherins, bearing homology to N- and P-cadherin, were found (Liaw et al., 1990). We have demonstrated, using a pan-cadherin antiserum, that the human endothelium contains three to four distinct cadherin-related bands, one of which was specifically identified as N-cadherin (Salomon et al., 1992) and one as cadherin-5 (Lampugnani et al., 1992).

Endothelial cell-cell adhesion mediated by molecules of the Ig superfamily was shown to occur via PECAM-1 (also known as CD31 or endoCAM) (Albelda et al., 1990, 1991; Newman et al., 1990; Simmons et al., 1990). While this molecule is apparently concentrated in cell-to-cell contact areas, its exact spatial, temporal, and functional relationship to cadherins is still not clear. It is noteworthy that in addition to endothelia, PECAM-1 is also present in platelets, monocytes, polymorphonuclear cells, neutrophils, and different subsets of T-cells (Muller et al., 1992; Newman et al., 1992; Tanaka et al., 1992).

The third adhesion system found in endothelia includes various members of the integrin superfamily (Lampugnani et al., 1991). These heterodimeric adhesion molecules are mainly involved in adhesion to extracellular matrix (ECM) components such as fibronectin and vitronectin, yet in endothelia, and in several other cell types, they were also localized to cell-cell adhesions. It is still unclear whether they directly mediate intercellular adhesion in these sites or interact indirectly via interlinking ECM molecules.

Our major objective, in this study, was to characterize the structural and functional interrelationships between the two major junctional systems present in the human endothelium, namely cadherins and PECAM-1. Using both conventional and three-dimensional digital microscopy, on double immunolabeled cultured human umbilical vein endothelial cells (HUVEC), we show that cadherin- and PECAM-1-mediated adhesions are differentially segregated along the lateral plasma membrane and display distinct modes of linkage to the cytoskeleton. Furthermore, short chelation of Ca^{2+} ions, selectively, disrupted the cadherin-mediated junctions leaving the PECAM-1 adhesions apparently intact. In light of these results we discuss the functional significance of the spatial segregation and adhesive intercellular mechanisms in endothelial cells.

Materials and Methods

Immunochemical Reagents

Pan-cadherin antibodies were prepared in rabbits by injections of a synthetic peptide corresponding to the 24 COOH-terminal amino acids of chicken N-cadherin coupled to hemocyanin. These antibodies were previously shown to react with essentially all known, full-length cadherins, in a wide variety of species (Geiger et al., 1990). Anti-human-N-cadherin (1447) was prepared in rabbits by multiple injections of a bacterial recombinant peptide corresponding to the extracellular portion of human N-cadherin, starting from the EC2 domain, up to the end of the cytoplasmic domain, excluding the eight most COOH-terminal amino acids (amino acid 319-897, see Salomon et al., 1992). Anti-PECAM-1 was a mouse monoclonal antibody raised against a gel-purified ~130-kD band from HUVEC. The hybridoma clone that was selected for the present study (3F12) was subsequently used to clone a PECAM-1-like c-DNA from human endothelial λ_{gt1} expression library. Immunofluorescence staining of HUVEC with this antibody was identical to that obtained with other anti-PECAM-1 monoclonal antibodies such as monoclonal antibody clones 9G11 and 1B5 (Lampugnani et al., 1992). The latter antibody was used for immunoelectron microscopy. Anti-cadherin-5 were mouse monoclonal antibodies clones 7B4 and TEA 1.31 prepared as previously described (Lampugnani et al., 1992). Anti-human-P-cadherin, raised in rabbits against a bacterial recombinant peptide, was kindly supplied by Dr. D. Vestweber (Max-Planck-Institute For Immunobiology, Freiburg, Germany). Anti-vinculin was a monoclonal antibody raised against human vinculin and purchased from Sigma Immunochemicals (St. Louis, MO). Anti-human-Von-Willerband Factor was a rabbit polyclonal antibody (BioMakor, Rehovot, Israel). Actin filaments were stained with FITC-phalloidin (Sigma Immunochemicals).

Fluorescein- and rhodamine-conjugated secondary antibodies (reactive with either rabbit or mouse IgG) were purchased from Jackson Labs. (West Grove, PA) and cross-adsorbed, whenever necessary, on the heterologous IgG to diminish cross-reactivity.

Cell Culture

Endothelial cells were harvested from human umbilical veins as previously described (Jaffe et al., 1972, 1973). Briefly, umbilical veins were cannulated, rinsed with PBS, then incubated with 0.5 mg/ml collagenase (CLS-1; Worthington Biochem. Corp., Freehold, NJ) for 30 min, and the detached cells plated on gelatin-coated culture plates (FALCON Labware, Meylan Cedex, France). The medium was M-199 with Earle's salts, buffered with 20 mM Hepes and supplemented with 100 μg/ml of bovine brain Endothelial Cell Growth Supplements, routinely isolated from bovine brains according to Maciag et al. (1979), heparin (100 μg/ml), 20% fetal calf serum (GIBCO BRL, Gaithersburg, MD), and antibiotics. Cells were fed every third day with fresh medium and split soon after reaching confluence by 2-min treatment with 0.1% trypsin. Cells used in this study were all of early (up to the third) passages and were routinely checked for the expression of Von-Willerband factor by immunofluorescence microscopy.

Other cells used in this study included CHO cells and A-431 cells, all originally obtained from the American Type Culture Collection (Rockville, MD). Transfection of CHO cells with human-N-cadherin was previously described (Salomon et al., 1992).

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1. *Abbreviations used in this paper*: AJ, actin-associated adherens-type junction; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells.

Indirect Immunofluorescence

Cells cultured on glass coverslips were fixed and permeabilized with a mixture of 3% paraformaldehyde and 0.5% Triton X-100 (2 min) and further fixed for 20 min with 3% paraformaldehyde alone. The cells were then rinsed and incubated for 45 min at room temperature with the relevant primary antibodies, washed three times with PBS, and incubated for 45 min with the fluorophore-conjugated secondary antibodies. Stained coverslips were mounted in elvanol (Mowiol 4-88; Hoechst, Frankfurt, Germany) and routinely examined with a Zeiss Axiophot microscope using a 100/1.3 Planapochromat objective. Photographs were taken using Tmax 3200 or 400 black and white films (Eastman Kodak, Rochester, NY).

Digital Immunofluorescence Microscopy and Three-dimensional Reconstruction

Cultured HUVEC were fixed and stained as described above. Stained coverslips were mounted in 90% glycerol (in 0.1 M Tris-Cl, pH 8.0), supported at their margins by #1 glass coverslips to avoid compression and improve the preservation of the three-dimensional structure of the cells.

The computerized microscopic system used here was based on the design of Agard and Sedat (Agard et al., 1988, 1989), consisting of a Zeiss Axiomat microscope and Microvax III workstation which controls image acquisition, light shutters, and focus. Images were recorded with a cooled, scientific-grade, charge-coupled device camera (Photometrics, Tucson, AZ). Images were read into an array processor (Mercury Computer Systems, Lowell, MA) which calculated on-the-fly pixel per pixel correction for illumination and charge-coupled device sensitivity, and scaled and deconvoluted images, essentially in real-time (Chen et al., 1990; Hiraoka et al., 1990). For cultured HUVEC, optical sections 0.2 μm apart were taken in the fluorescein and rhodamine channels with selective filter sets.

The three-dimensional imaging and modeling package used for image reconstruction (Chen et al., 1990; Kam et al., 1991) employs a high-resolution (1280 × 1024, 60 Hz, noninterlaced) video board with 12 Mbytes of memory (PRISM; Parallax Graphics, Inc., Santa Clara, CA), and implements a flexible multiple windowing architecture and interactive display of image series. Versatile color menus allow images of different fluorophore staining of the same sample to be superimposed. Pairs of images from tilt projection series (±10°) were selected for stereo presentations in this work. Pictures were photographed on Ektachrome 160 film (Eastman Kodak) using Focus 4700 Imagecorder (Focus Graphics, Foster City, CA).

Electron Microscopy

Human placenta was obtained immediately after delivery and fixed for 3 h with 4% paraformaldehyde. Blood vessels were gently dissected, embedded in 10% gelatin (30°C, 15 min), and refixed for 20 min. The tissue was infiltrated with 2.3 M sucrose and ultrathin (800–1000 Å) sections cut at −105°C in an ultramicrotome (FC/4D; Reichert, Wien, Austria). Sections were incubated with either the pan-cadherin or anti–PECAM-1 antibodies. The labeling of the latter was enhanced by additional incubation with rabbit anti–mouse antibodies (ZYMED Lab., San Francisco, CA), followed by 10 nm gold conjugated goat anti–rabbit IgG (ZYMED Lab.). The sections were then stained with 3% uranyl acetate and mounted in 2% methylcellulose according to Tokuyasu and Singer (1976). The sections were examined using a Philips CM12 electron microscope at 100 KV.

Selective Extractions with Triton X-100

Confluent HUVEC cultures were washed three times with 50 mM MES buffer, pH 6.0, followed by 5-min extraction with 0.5% Triton X-100 in the same buffer at 4°C. The Triton-soluble fraction was centrifuged for 15 min at 10000 g and the supernatant was mixed with 3× sample buffer (Laemmli, 1970). The Triton-insoluble fraction was washed with PBS and extracted with sample buffer. For immunofluorescence analysis, cells cultured on glass coverslips were extracted with 0.5% Triton X-100 in 50 mM MES buffer for 3 min, fixed for 20 min with 3% paraformaldehyde, and immunolabeled for cadherins and PECAM-1.

Electrophoresis and Immunoblotting

Cells were homogenized directly in boiling laemmli sample buffer and subjected to SDS-PAGE (Laemmli, 1970) on 4% polyacrylamide gels followed by electrophoretic transfer of the proteins to nitrocellulose paper (Hybond™-C; Amersham International PLC, Buckinghamshire, England).

The positions of polypeptide bands were identified by immunoblotting with corresponding antibodies, followed by horseradish peroxidase-linked goat anti–mouse Ig or protein-A. Bands were detected using the ECL™ Western blotting detection system (Amersham International PLC).

EGTA Treatment

Cells cultured on glass coverslips were washed three times with Ca^{2+}-free PBS and incubated with 3 mM EGTA in PBS (Ca^{2+}-free) for 5 min. Cells were then fixed and stained as described above.

Results

Adhesion Molecules of Cultured Human Endothelial Cells

Immunoblotting analysis of HUVEC extracts using the pan-cadherin antibodies, revealed four immunoreactive bands in the relevant molecular weight range (Fig. 1 a). These include
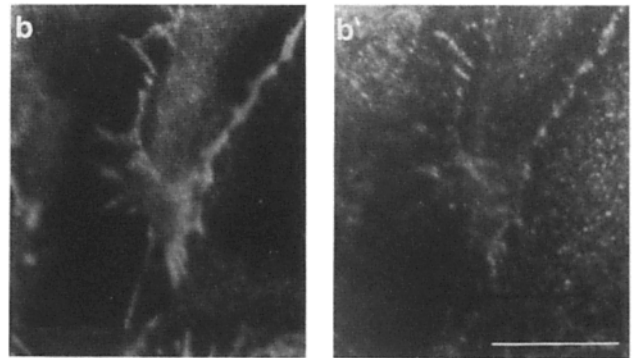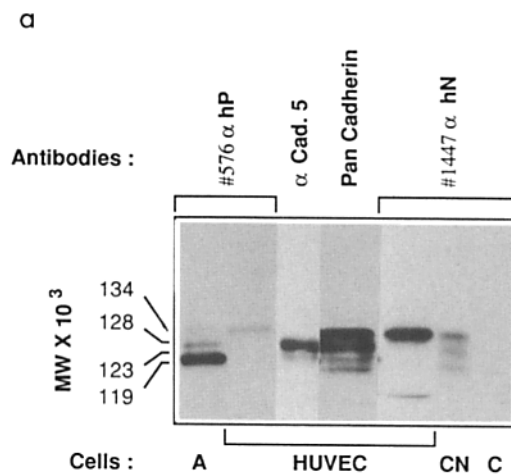

Figure 1. (a) Immunoblotting analysis of extracts of cultured HUVEC (*HUVEC*), A-431 cells (*A*), CHO cells transfected with human N-cadherin cDNA (*CN*), and nontransfected CHO cells (*C*) using the pan-cadherin antibodies (*Pan Cadherin*), cadherin-5 specific antibodies (α *Cad.5*), N- and P-cadherin- specific antibodies (*#1447* α *hN* and *#576* α *hP*, respectively). Note that HUVEC contain four pan-cadherin–reactive bands including N-cadherin and cadherin-5. Double immunolabeling of HUVEC with pan-cadherin antibodies (*b*) and anti cadherin-5 (*b'*). Notice that cadherin-5 is partially colocalized with the cadherin-positive area. Bar, 10 μm.

two major bands, with apparent molecular masses of 135 and 130 kD, and two minor bands of ~125 and 120 kD (Fig. 1 a, HUVEC, Pan Cadherin). The upper (135 kD) band was identified as N-cadherin, based both on its comigration with N-cadherin in transfected CHO cells, and on its interaction with anti-human N-cadherin antiserum (Fig. 1 a). The 130-kD band was identified as cadherin-5 (Suzuki, 1991) using specific antibodies (Fig. 1 a, HUVEC, αCad.-5). The exact identity of the other two bands is not clear. The 125-kD band apparently comigrated on SDS gels with human P-cadherin from cultured A-431 cells (recognized both by pan-cadherin and anti P-cadherin antibodies [Fig. 1 a, A, Pan-Cadherin, and αhP-cad., respectively]). However, the same anti P-cadherin did not react specifically with respective band in the HUVEC extract (Fig. 1 a, HUVEC, αhP-cad.). Therefore, the nature of this as well as of the 120-kD band is still unclear.

Double immunofluorescent labeling of HUVEC using the pan-cadherin antibodies and anti–cadherin-5 revealed that both antibodies reacted with intercellular AJs (Fig. 1, b and b', respectively), yet the staining was clearly distinct. The cadherin-5 antibodies (b') labeled only part of the entire junctional structure, as seen by the pan-cadherin antibodies (b).

Immunoblotting with anti PECAM-1 antibodies consistently revealed two major immunoreactive bands (~126 and 124 kD) in HUVEC extract (Fig. 7 c), suggesting the presence of more than one form of this molecule.

Cellular Distribution of Cadherins, PECAM-1, and AJ Cytoplasmic Components in Human Endothelial Cells

Cultured HUVEC form a confluent monolayer consisting of closely associated cells. To determine the spatial relationships between either cadherins or PECAM-1 and cell-to-cell AJ, the fixed and permeabilized monolayers were double labeled for either adhesion molecule in conjunction with AJ-
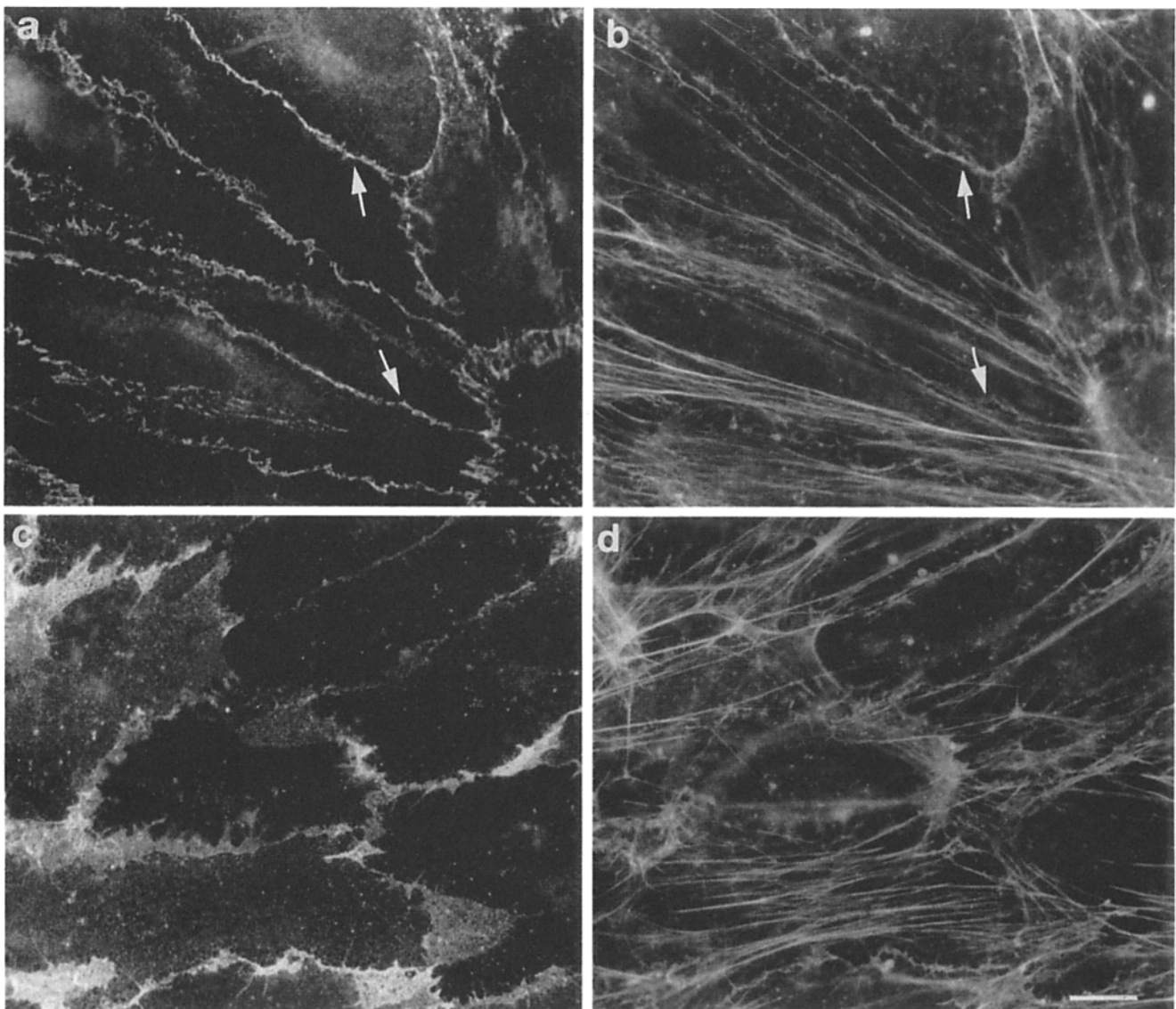

Figure 2. Double immunofluorescence labeling of cultured HUVEC with Pan-cadherin (a) or PECAM-1 antibodies (c) and for actin (b and d, respectively). Notice that junction-bound actin is closely associated with cadherins (matching arrows in a and b) while PECAM-1 is located in more basal areas (see below) and its distribution does not overlap with that of junctional actin. Bar, 10 μm.
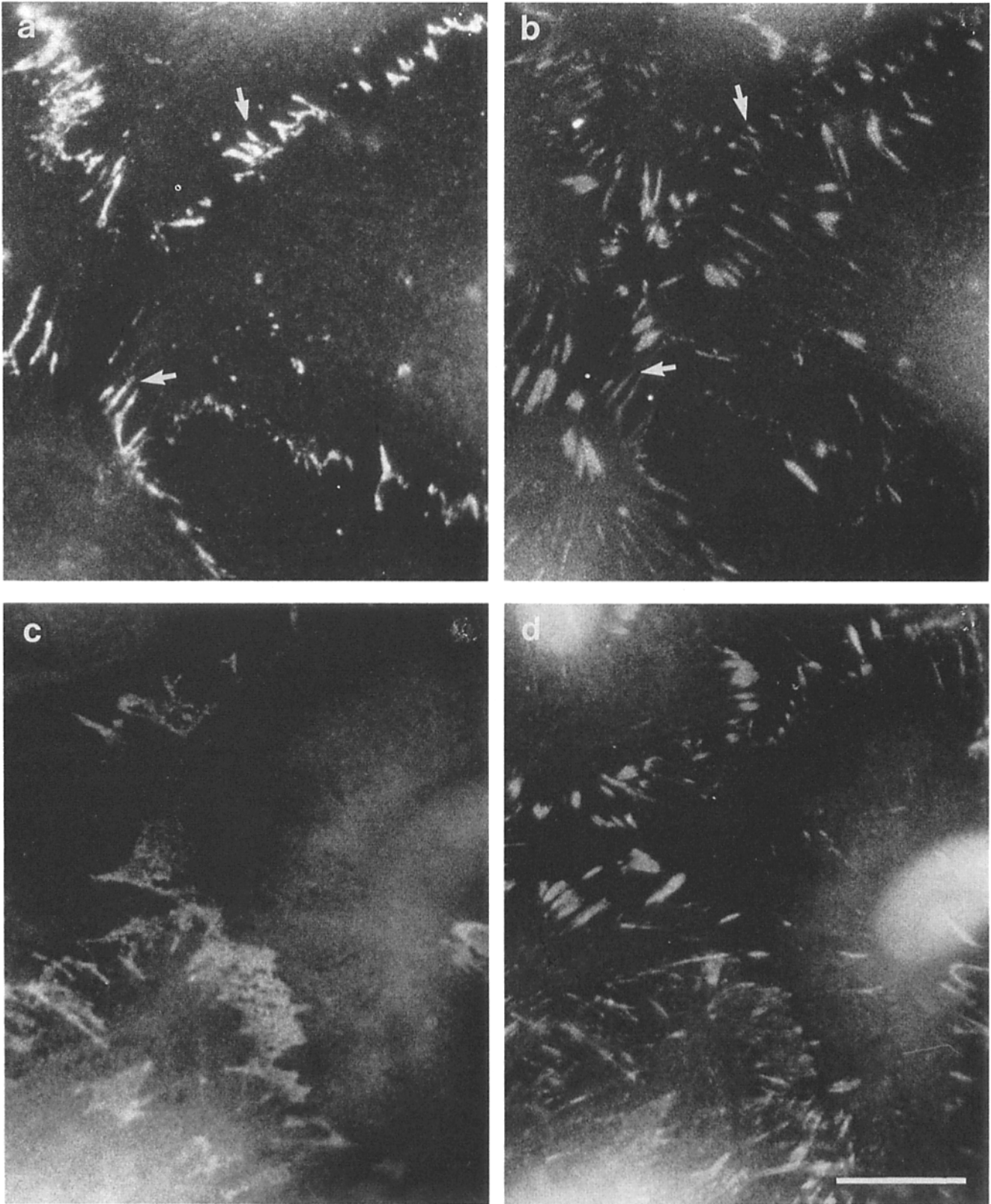
Figure 3. Indirect double immunofluorescence labeling of HUVEC, comparing the cellular distribution of vinculin (*b* and *d*) with that of cadherins (*a*) and PECAM-1 (*c*). Note the close similarity between the distributions of cadherins and vinculin, as indicated by the matching arrows in *a* and *b*. In contrast, vinculin-rich focal adhesions, which are very prominent in HUVEC, do not contain PECAM-1. The distribution of PECAM-1 is characteristically less focal than that of vinculin and is often localized in different sites (compare *c* to *d*). Bar, 10 μm.

specific molecules, such as actin and vinculin (Figs. 2 and 3). As depicted in Fig. 2, b and d, actin bundles in these cells were either radial or circumferential, forming a distinct band along cell–cell contacts. Comparison of this pattern to that of cadherins (Fig. 2 a) and PECAM-1 (Fig. 2 c) revealed a significant difference: cadherins were closely related to actin microfilaments at junctional sites, whereas PECAM-1 distribution in these regions was clearly distinct, and usually less focused.

This difference was further corroborated by comparing the subcellular distribution of cadherins or PECAM-1 with that of vinculin (Fig. 3). Vinculin is abundant in HUVEC and is associated both with ventral focal contacts and with cell–cell

AJ (Fig. 3, b and d). This junctional vinculin, which was located at variable focal planes along the lateral membrane, displayed close association with cadherins (compare Fig. 3, a and b). In contrast, the distributions of PECAM-1 and vinculin were adjacent, yet clearly distinct (compare Fig. 3, c and d).

Spatial and Temporal Inter-relationships Between Cadherins and PECAM-1

To directly determine the differential distributions and spatial inter-relationships of cadherins and PECAM-1, confluent HUVEC monolayers were double labeled with pan-cadherin antiserum and anti PECAM-1 mAb (3F12). The immunoflu-
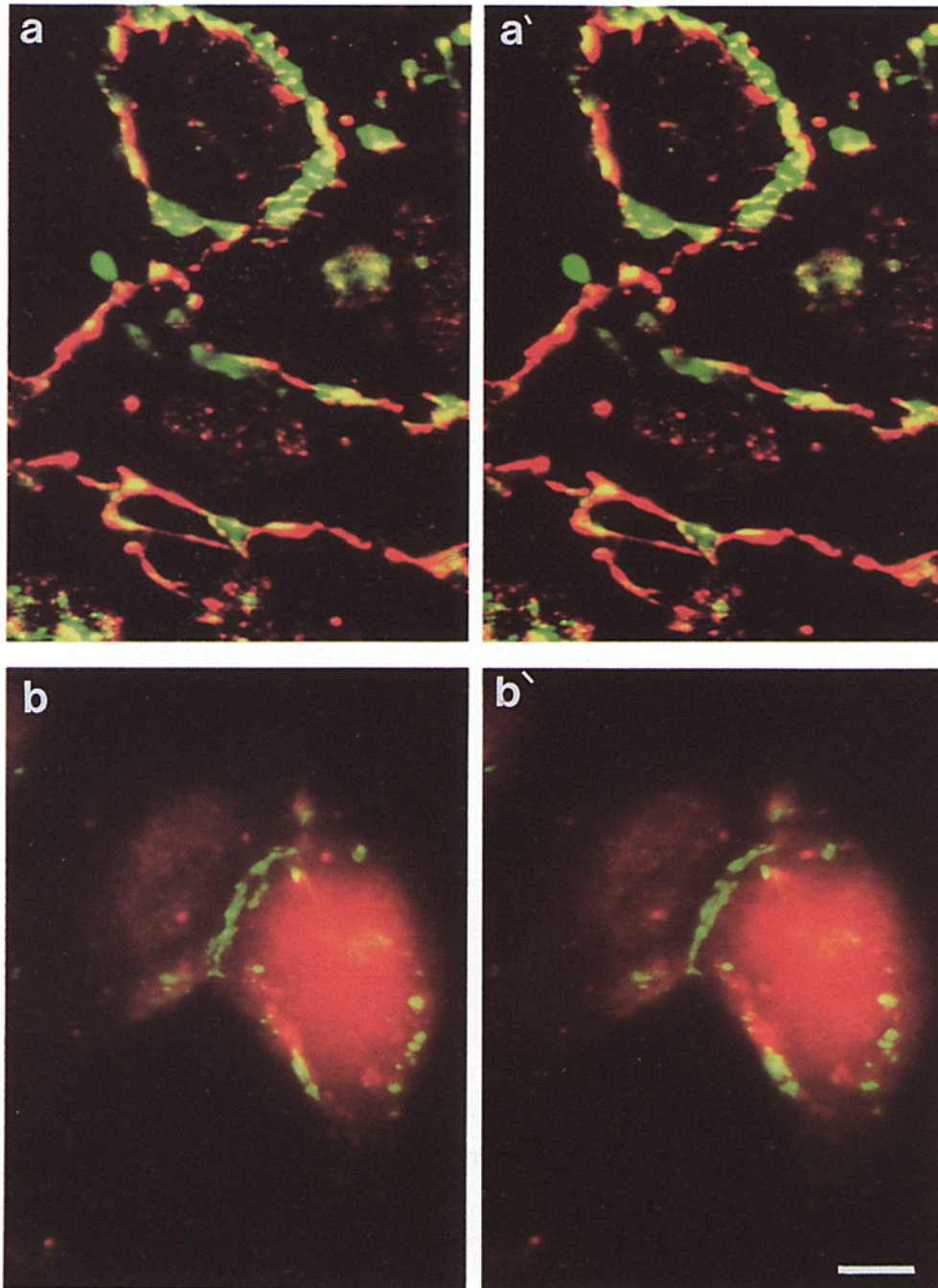

Figure 4. Double immunofluorescence labeling of control HUVEC (a) as well as of HUVEC which were briefly treated with 3 mM EGTA (b). The cells were fixed and labeled with pan-cadherin antibodies (red) and with anti PECAM-1 (green). The cells were examined by digital microscopy and 3-D images reconstructed. Pairs of images from a tilt projection series (±10°) were selected for stereo presentations (a and b). Bar, 10 μm.
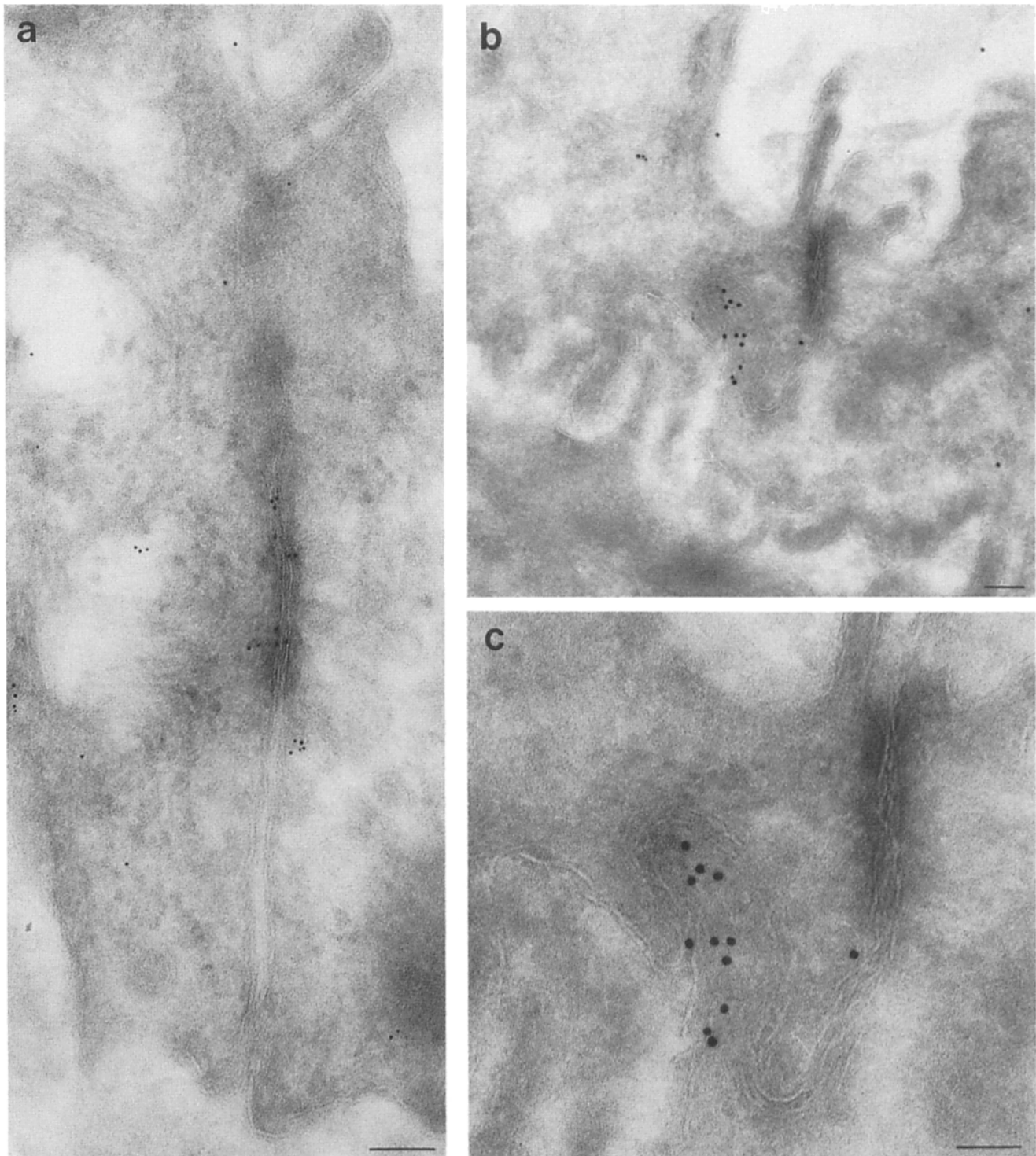
Figure 5. Immunogold labeling of ultrathin frozen sections of freshly fixed human placental blood vessels. Sections were labeled with pan-cadherin antibodies (*a*) or with PECAM-1–specific antibodies (*b* and *c*). Cadherin molecules were found mostly in subapical, electron-dense areas, with a typical appearance of cell–cell AJ. PECAM-1, on the other hand, was located at more basal positions along the lateral plasma membrane (*b*) and was not associated with the dense subapical region (*c*).

orescent patterns were analyzed by the digital microscopic system, based on the recording of serial optical sections 0.2 μm apart. As shown in Fig. 4 *a*, the distribution of the two adhesion molecules was close but clearly distinct, in line with the colocalization of each molecule with actin and vin-culin (Figs. 2 and 3). Generally PECAM-1 (Fig. 4 in green) was located at more basal positions than cadherin along the lateral plasma membrane (upper part of Fig. 4 *a*). The distance between the cadherin and PECAM-containing adhesion sites was typically 0.4 μm or more (see deeper part in

Fig. 4 a). This difference in the relative localization of cadherins and PECAM-1 was corroborated by immunoelectron microscopy (Fig. 5). Immunogold labeling of ultrathin frozen sections of freshly fixed human placental blood vessels, indicated that cadherin molecules were concentrated at a subapical, electron-dense area of the cells, along structures with a typical appearance of cell-cell AJ (Fig 5 a). PECAM-1, on the other hand, was located at more basal positions along the plasma membrane (Fig. 5 b) and was not detected in the AJ-associated electron-dense area (Fig. 5 c). This observation, made here with intact tissues is in general agreement with the immunofluorescence data, obtained with cultured HUVEC (Fig. 4, a and a'). Attempts to carry out simultaneous immunoelectron microscopic localization of the two proteins were not successful, due to the limited sensitivity of labeling without amplification (which is not compatible with double labeling).

In order to compare the temporal relationships between the two adhesion systems, tripsinized HUVEC were plated on cover slips at high density and the distribution of cadherins and PECAM-1 were examined at relatively short intervals after plating. As shown in Fig. 6, cadherins first appeared as patches at nascent contact sites 2-3 h after plating (Fig. 6 a). PECAM-1, on the other hand, was not organized in discrete junctions at this stage (Fig. 6 b), and appeared in cell-cell contact sites only 4-6 h after plating. The slower assembly of PECAM-1 could not be attributed to preferential sensitivity of this molecule to trypsin since Western blot analysis indicated that PECAM-1 levels fully recovered by 3 h after replating. The nascent PECAM-1 patches were commonly distributed in areas where cadherins were already well organized (compare Fig. 6, c and d).

Differential Effect of Ca^{2+} Chelation on Cadherin- and PECAM-1-mediated Adhesions

To study the functional interdependence between the cadherin- and PECAM-1-containing adhesions we have briefly treated HUVEC with EGTA, which is known to specifically perturb epithelial junctions, and AJ in particular (Volberg et al., 1986; Volk et al., 1990). As shown in Fig. 4 b, short EGTA treatment resulted in a dramatic disruption of cadherin labeling (red), leading to its dispersal throughout the cell surface. On the other hand, the distribution of PECAM-1 (green) was largely unaffected by this treatment (Fig. 4 b). The chelation of Ca^{2+} ions was often accompanied by some detachment of cells from the substrate and the dispersal of cadherin was nearly complete in relatively sparse regions
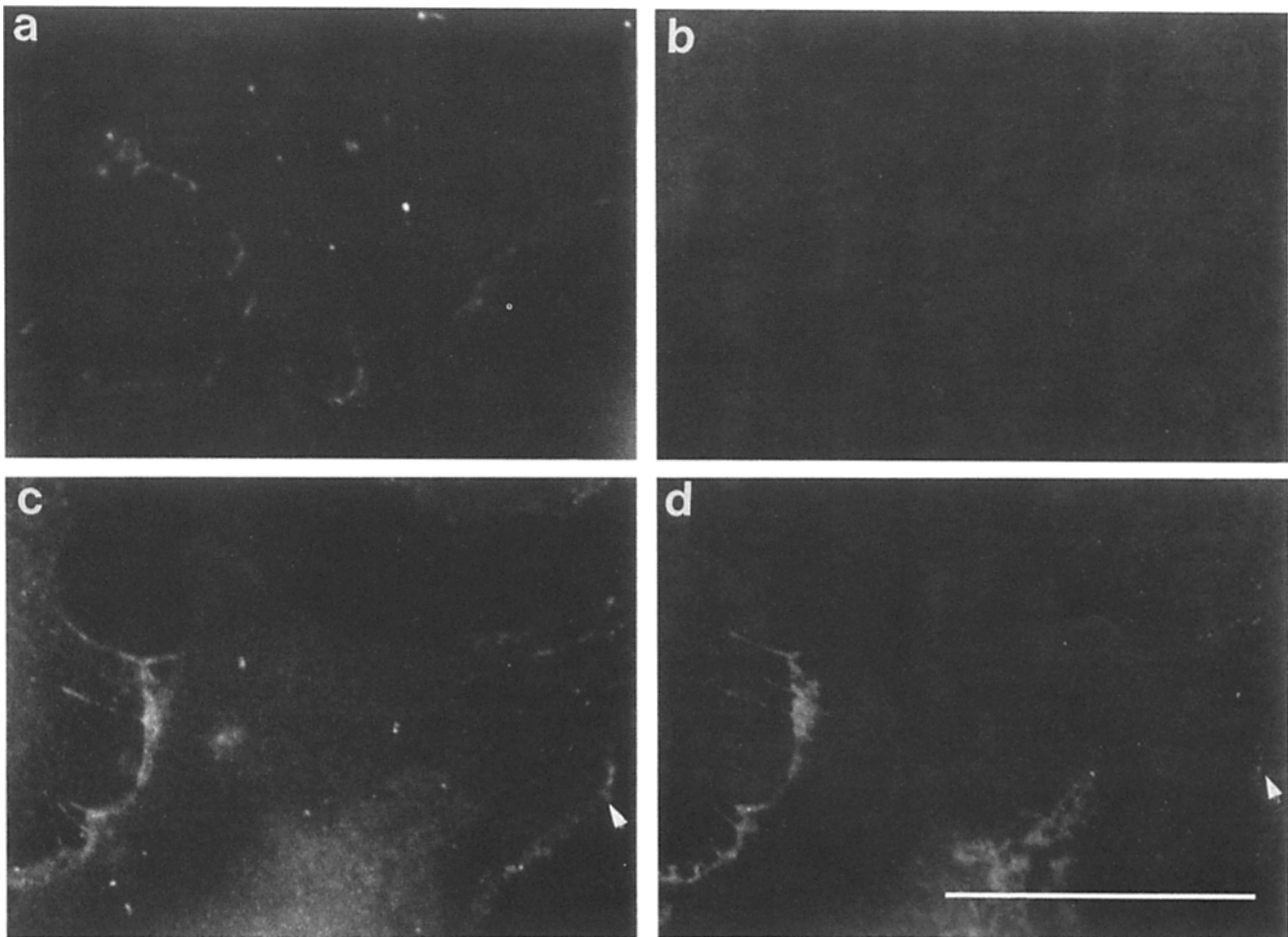

Figure 6. Double immunofluorescence labeling of densely plated HUVEC for cadherins (a and c) and PECAM-1 (b and d). The distributions of the two molecules were examined 3 h (a and b) and 4 h (c and d) after seeding. Arrowheads point to PECAM-1 containing sites along cadherin-rich adhesions. Bar, 10 μm.

254

along the culture. Furthermore, the brief incubation with EGTA was accompanied by a moderate rounding up of the treated cells.

Differential Association of Cadherins and PECAM-1 with the Cytoskeleton

To determine whether the above-demonstrated structural and functional differences between cadherin- and PECAM-1-mediated junctions reflect differences in the linkage of the two systems to the cytoskeleton, we examined the differential resistance of the two molecules to detergent extraction. As depicted in Fig. 7 *a*, extensive cadherin labeling was associated with cell–cell contacts, following Triton X-100 extraction, whereas PECAM-1 was essentially completely extracted (Fig. 7 *b*). The distributions of actin microfilaments and of vinculin were not affected by this treatment and they remained apparently associated with cadherins (not shown). To quantitatively determine the differential detergent solubility of the two adhesion molecules, we analyzed the Triton X-100 soluble and insoluble fractions by Western blotting, followed by densitometric scan of the bands. As shown in Fig. 7, *c* and *d*, over 85% of the cadherins were found in the Triton-insoluble fractions. This refers mainly to the higher molecular mass cadherin bands, since the lower bands (125 and 120 kD) were not readily detectable either in the Triton-soluble or -insoluble fractions. PECAM-1, on the other hand, was mainly (>70%) found in the Triton-soluble fraction (Fig. 7, *c* and *d*).

Discussion

Endothelial cells both in culture or in situ, contain multiple intercellular contact receptors which are responsible for the formation and maintenance of the endothelial layer (Newman et al., 1990; Simmons et al., 1990; Albelda et al., 1991; Lampugnani et al., 1991, 1992; Salomon et al., 1992). Based on sequence homology and immunoreactivity, these molecules were classified into three major superfamilies, namely cadherins, integrins, and immunoglobulins (for reviews see Albelda and Buck, 1990; Albelda, 1991; Wheelock and Knudsen, 1991; Geiger and Ayalon, 1992; Hynes and Lander, 1992). Members of another group of adhesion molecules, namely the selectins, are present in endothelia, yet are predominantly implicated in the recruitment of leukocytes to the endothelial surface (Lasky, 1991; Spertini et al., 1991; Tyrrell et al., 1991; Ley, 1992). The activity of each of the junctional receptors was previously investigated in a large variety of systems, yet their spatial and temporal relationships as well as their differential roles and coordinated action in mediating endothelial adhesion is still poorly characterized.

In this study we have examined the spatial distributions of two adhesion systems, namely PECAM-1 and cadherins, in cultured human endothelial cells, and compared some of their functional properties. The study focused only on direct homotypic intercellular adhesion mechanisms (namely adhesion between cells of the same type) and did not address the other adhesive systems which are responsible primarily
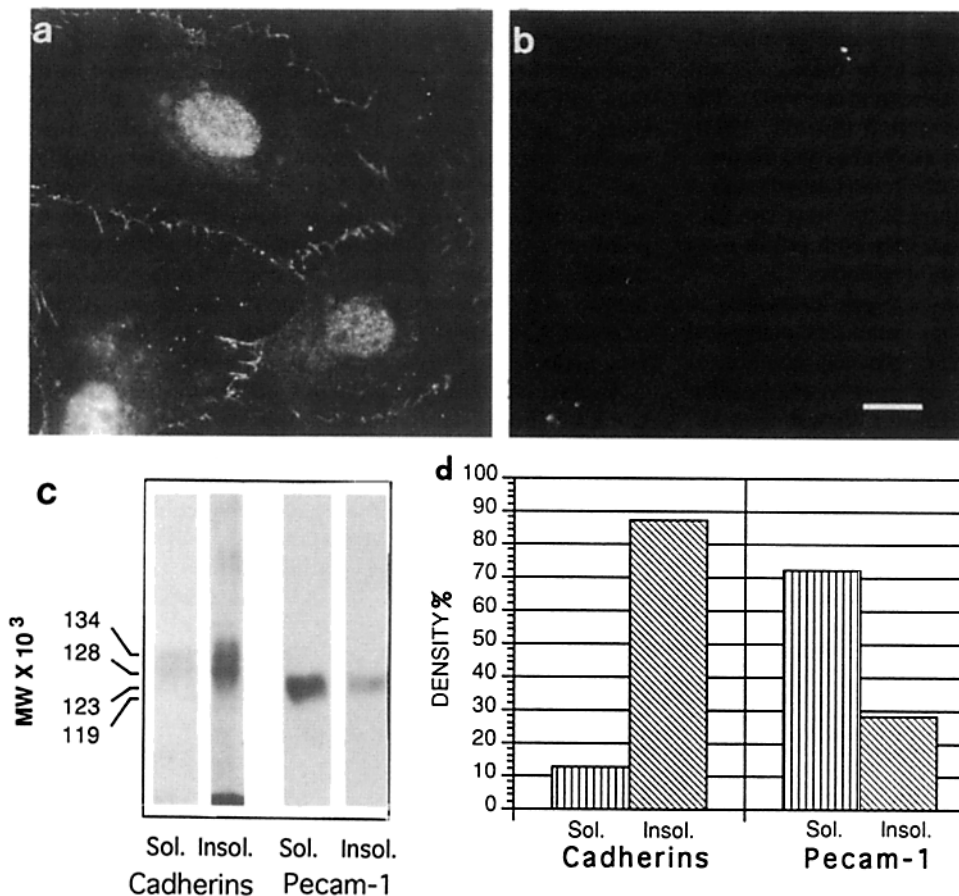

Figure 7. Double immunofluorescence labeling of HUVEC, which were extracted with Triton X-100, for cadherins (*a*) and PECAM-1 (*b*). Quantitative analysis of the Triton-soluble and -insoluble fractions was performed using Western blotting, following labeling with pan-cadherin antibodies (*c, Cadherins*) or with PECAM-1-specific antibodies (*c, Pecam-1*). The intensity of each band was determined by densitometric scanning of the bands. As shown (*d*), over 85% of the cadherins were retained in the Triton-insoluble fractions (*d, Cadherins*), while PECAM-1 was mainly (>70%) found in the Triton-soluble fraction (*d, Pecam-1*). Bar, 10 µm.

for the binding to leukocytes or platelets (Bochner et al., 1991; Dobrina et al., 1991; Lauri et al., 1991; Letts and Liu, 1991; Haskard and Lee, 1992). In addition, we did not examine here the involvement of integrins (namely $\beta_1\alpha_2$, $\beta_1\alpha_5$, and α_v integrins), which are primarily associated with matrix adhesions, yet are also present in endothelial cell–cell adhesions (Lampugnani et al., 1991). It is still unclear whether these receptors indeed directly mediate intercellular adhesion or interact indirectly via ECM molecules.

The fact that multiple adhesion systems may coexist in the same cells has been known for a long time, for example the presence of N-CAM and N-cadherin in neural tissues (Doherty et al., 1991; Doherty and Walsh, 1991) or of PECAM-1 and cadherins in endothelia (Albelda et al., 1990; Albelda, 1991; Lampugnani et al., 1992; Muller et al., 1992), nevertheless the functional interrelationships between such systems is not clear. Conceptually, the various adhesion molecules could coassemble into the same junction, thus showing an extensive overlap in their distribution. Alternatively, each adhesion system could develop separately and their components might display a mutually exclusive distribution. Moreover the specific relationships might change at different phases of junction formation.

It should be mentioned that the diversity of adhesion molecules in endothelial junctions is much larger since each superfamily of adhesion molecules is represented by more than one isoform. The presence, in human endothelium, of multiple cadherins was previously reported (Salomon et al., 1992) and extended in this study. Among the four pan-cadherin-reactive bands, the largest polypeptide (135 kD) was identified as N-cadherin, based on its comigration with the band found in CHO cells transfected with the respective cDNA and its reactivity with anti-human N-cadherin–specific antibodies. Moreover, expression of N-cadherin by these cells was verified by northern blot analysis (Salomon et al., 1992). The 130-kD band was identified as cadherin-5 (Suzuki, 1991), based on its interaction with specific antibodies and the presence of the respective RNA in endothelium (Lampugnani et al., 1992). The exact molecular nature of the other two cadherin-related bands is not clear since they both fail to react with P-, E-, and N-cadherin–specific antibodies.

In this study we have used pan-cadherin antibodies to localize AJ. This is based on previous immunocytochemical studies, which showed that different cells may contain, as their transmembrane AJ elements, different types of cadherins including E-cadherin (uvomorulin) (Vestweber et al., 1987), N-cadherin (Hatta and Takeichi, 1986; Volk and Geiger, 1986; Reid and Hemperly, 1990; Walsh et al., 1990; Salomon et al., 1992), or P-cadherin (Nose et al., 1987; Hirai et al., 1989; Shimoyama et al., 1989). The use of the pan-cadherin antibody, rather than antibodies specific for each of the various known cadherins has the advantage that it may overlook variations in the distributions of individual cadherins and serve as a specific, yet general marker of AJ. Furthermore, the association of pan-cadherin labeling with AJ was corroborated by its colocalization with junctional vinculin and actin (Figs. 2 and 3), as well as by its immunoelectron microscopic reactivity (Fig. 5).

The preferential association of cadherins with AJ is also supported by the differential resistance of cadherins and PECAM-1 to detergent extraction. As shown above, over 85% of the cadherins were retained, following Triton X-100 extraction, along with the cytoskeleton, whereas nearly 70% of PECAM-1 was extracted by the detergent. This conclusion was confirmed by the immunofluorescence labeling which pointed to a selective retention of cadherins with the Triton-insoluble cytoskeleton.

Cell adhesion between endothelial cells, driven by adhesion molecules of the immunoglobulin superfamily, was represented in this work by PECAM-1 (Newman et al., 1990; Simmons et al., 1990; Valent et al., 1990; Albelda et al., 1991). This molecule may play a dual role, being present on both endothelial cells and on leukocytes; it could function to heterotypically bind the latter to the vessel wall or to mediate homotypic interactions between the endothelial themselves. It should be mentioned that PECAM-1 appears in HUVEC and in platelets as two distinct bands (Newman et al., 1992; Albelda et al., 1991, and Fig. 7 c). It is still not clear whether the two are products of distinct or alternatively spliced genes or one is a phosphorylated derivative of the other. Additional studies will be necessary to find out the molecular basis for this diversity and its functional significance.

The spatial hierarchy of AJ and PECAM-1, which was demonstrated in cultured HUVEC by three-dimensional microscopy, indicated that the two were located at the vicinity of each other, yet were clearly distinct. Moreover, the cadherin-containing AJ were generally located at more apical positions than PECAM-1, along the lateral cell membrane.

While the distinct distributions of PECAM-1 and cadherins indicate that the two do not coassemble and intermix, the close proximity of the respective junctions suggests that the two assemble in a coordinated fashion or are conucleated. This is supported by double labeling of HUVEC at relatively short intervals after plating, demonstrating that cadherins became organized in surface patches much earlier than PECAM-1. When PECAM-1 first appeared at the cell surface, it was invariably associated with cadherin-rich regions suggesting that nascent cadherin-containing AJ serve as the nucleation sites for subsequent and adjacent assembly of PECAM-1 adhesions. This does not exclude the possibility that the further development of a "mature" endothelial junction depends on the concerted interactions of the two adhesive systems, as indicated by the inhibitory effect of endoCAM antibodies on intercellular associations of bovine endothelial cells (Albelda et al., 1990).

Another general concept, highlighted by the present study, is the functional significance of multiple molecular systems mediating adhesion between the same cells. A possible explanation might be that such molecular diversity may play a role in securing stable and long term intercellular adhesion under changing environmental conditions. Thus external perturbations which affect only one of these adhesions may exert only limited effect on the coherence of the entire monolayer due to the presence of the second "backup system." This possibility is in line with the demonstration, here, that selective perturbation of AJ (by treating the cells briefly with EGTA) leads to essentially complete dispersal of cadherins, leaving the PECAM-1 distribution largely unaffected. This view is also supported by a recent report showing that addition of cadherin-5 monoclonal antibodies to the incubation medium, increases endothelial permeability without affecting the gross distribution of junctional molecules (Lampugnani et al., 1992).

It is noteworthy that the calcium independence of PECAM-1 has been recently questioned, since transfection of the respective cDNA resulted in the acquisition of calcium dependent cell–cell aggregation in the transfected cells (Albelda et al., 1991). It is, however, still unclear whether this calcium requirement is attributable to PECAM-1 alone or to heterophilic interactions of PECAM-1 with other ligands (Muller et al., 1992). Moreover, PECAM-dependent aggregation of transfected cells was shown to be divalent (mainly Mg^{2+}) cation dependent. In the present study, we used EGTA, which is specific to Ca^{2+} ions, and observed little effect on PECAM-1 junctions, supporting the view that the PECAM-mediated adhesion system is largely Ca^{2+} independent (Newman et al., 1990; Xie and Muller, 1993).

Further studies are required to extend our understanding of the physiological role of each adhesion system, and the ways in which these spatially distinct adhesive systems are temporally coordinating in response to changing conditions at a blood vessel wall.

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