Journal of Cell Science 109, 2141-2149 (1996)
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JCS9479

Desmoplakin expression and organization at human umbilical vein endothelial cell-to-cell junctions

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SUMMARY

Desmoplakin is an intracellular component of desmosomes which plays a role in the anchorage of intermediate filaments to these structures. We report here that, despite the absence of desmosomes, cultured endothelial cells from human umbilical vein express desmoplakin I and II both at mRNA and protein level. Desmoplakin I/II are found only in the detergent insoluble fraction suggesting that most of the protein is linked to the cytoskeleton. Desmoplakin I/II could be detected by western blot only in long confluent cells even if desmoplakin mRNA levels are unchanged by cell confluency. This suggests that desmoplakin might be stabilized at protein level by its association with junctional components. Immunofluorescence confocal microscopy showed that desmoplakin codistributes with VE-cadherin and plakoglobin along the lateral cell membrane. In contrast, desmoplakin localization was distinct from that of PECAM, an endothelial specific junctional protein localized outside adherence junctions. Endothelial cells do not have keratins but they express vimentin. In confluent cells vimentin forms peripheral filaments which attach to the cell membrane in areas at desmoplakin localization. These data suggest that desmoplakin may participate in the molecular organization of interendothelial junctions by interacting with VE-cadherin and promoting vimentin anchorage. This new type of intercellular junction seems to correspond to the 'complexus adhaerentes' described in vivo in lymphatic endothelium.

Key words: Desmoplakin, Endothelium, Adherence junction, Cadherin, Catenin

INTRODUCTION

Endothelial intercellular junctions are important structural determinants of vascular permeability, leukocyte extravasation and vascular remodeling (Franke et al., 1988; Dejana et al., 1995). Intercellular junctions are, in general, complex structures formed by transmembrane adhesive molecules responsible for the attachment between contiguous cells and an intracellular undercoat of cytoplasmic/cytoskeletal proteins which stabilizes the junctions and possibly mediates intracellular signalling (Geiger and Ayalon, 1992; Tsukita et al., 1992; Kemler, 1993; Grunwald, 1993; Hinck et al., 1994; Hülsken et al., 1994). On the basis of structural and functional characteristics at least three types of junctions have been described in endothelial cells. These are: tight junctions (Anderson et al., 1993; Gumbiner, 1993), adherence junctions (AJ) (Rubin, 1992) and gap junctions (Beyer, 1993).

AJ are formed by cadherins as transmembrane glycoproteins which promote homophilic, Ca^{2+} dependent cell-to-cell recognition. Endothelial cells have been found to express both specific (VE-cadherin) (Suzuki et al., 1991; Lampugnani et al., 1992) and non specific cadherins (N-cadherin and P-cadherin) (Rubin, 1992). VE-cadherin is ubiquitarious, i.e. it is expressed in all type of endothelium and is consistently located at AJ (Lampugnani et al., 1992). VE-cadherin, similar to the other members of the family (Geiger and Ayalon, 1992; Tsukita et al., 1992; Grunwald, 1993; Kemler, 1993), forms complexes with catenins (α-, β-catenins, plakoglobin and p120) and is indirectly associated with actin microfilaments (Lampugnani et al., 1995). Endothelial cells do not have structures similar to desmosomes. These additional types of intercellular junctions mediate adhesion between epithelial cells and are formed by specific transmembrane glycoproteins related to the cadherin family (named desmocollins and desmogleins) and by intracellular proteins such as plakoglobin and desmoplakin (DP) (for review see Schmidt et al., 1994). From in situ histological studies (Schmelz and Franke, 1993) endothelial cells do not express desmogleins or desmocollins. They do, however, express plakoglobin (Franke et al., 1988; Dejana et al., 1995) and in the lymphatic endothelium also desmoplakin (Schmelz and Franke, 1993; Schmelz et al., 1994).

In epithelial cells DPI and II (DPI/II) are the major components of desmosomes. The two proteins are encoded by a single gene, DPII message is derived from splicing of the original

transcript (Green et al., 1990; Virata et al., 1992). DPI is a homodimer of about 250 kDa with a central rod domain composed of an α helical coiled-coil flanked by amino and carboxyl-terminal globular domains. DPII has a molecular mass of about 220 kDa and a shortened rod domain (O'Keefe et al., 1989; Green et al., 1990).

In desmosomes, DP is linked, directly or indirectly, with plakoglobin, and the carboxyl terminus of DP is thought to be a potential site for ionic interaction with intermediate filaments such as keratin and vimentin (Stappenbeck and Green, 1992; Stappenbeck et al., 1993).

In order to further characterize the molecular components of interendothelial clefts, with immunofluorescence we screened a series of antibodies directed to typical components of epithelial junctions. Surprisingly we found that human umbilical vein endothelial cells were positively stained by a DP polyclonal antiserum. This observation prompted us to investigate in more detail whether umbilical vein endothelium expresses DP. The results indicate that DPI/II are indeed present in cultured human umbilical vein endothelial cells (HUVEC) both at mRNA and protein levels. DP appears to codistribute with AJ components such as VE-cadherin and related catenins (plakoglobin, β-catenin and p120) at the most basal site of intercellular junctions. These data confirm and extend the in vivo observations of Schmelz et al. (1994; Schmelz and Franke, 1993) and further support the concept of the presence in the endothelium of a new type of DP containing AJ.

MATERIALS AND METHODS

Cell culture

HUVEC were cultured and characterized as described before (Lampugnani et al., 1992). Cells were cultured on plastic flasks coated with 1% gelatin. For immunofluorescence studies, cells were grown on glass coverslips (12 mm diameter) coated with human plasma fibronectin (7 μg/ml) (Boehringer, Mannheim, Germany). Junction organization and DP distribution were studied at different cell densities. HUVEC were seeded at two initial densities to reach different stages of confluence at the same time after seeding in culture (72-96 hours). Subconfluent cells (seeding density 1.2×10^3 cell/cm^2) presented sparse cell contacts at the time of the experiment (cell density was $5\text{-}6\times10^3$ cells/cm^2). Long confluent cells (seeding density 10×10^3 cell/cm^2) reached confluence 48-72 hours prior to the experiment. The cells showed limited spreading and reached high density ($56\text{-}65\times10^3$ cells/cm^2).

In other experiments, confluent HUVEC were treated for 24 hours with: endotoxin (LPS, 10 μg/ml) from Sigma (St Louis, MO, USA), transforming growth factor (TGF) β1 (20 ng/ml) from Genzyme (Cambridge, MA, USA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) (10 ng/ml) from Sigma.

Cell lines HeLa (clone CCL-2), HEL (clone TIB-180) (Martin and Papayannopoulo, 1982), HL60 (clone CCL-240) (Collins et al., 1977) and MOLT4 (clone CCL-1582) were obtained from the American Type Culture Collection, and NB4 (Lanotte et al., 1991) from Pasteur Institute. These lines were cultured in RPMI 1640 (Gibco, Cergy-Pontoise, France) supplemented with 10% inactivated fetal calf serum (FCS) (Gibco).

Polymerase chain reaction (PCR), cDNAs cloning and sequence analysis

Total RNAs were prepared from various cell types using a guanidium isothiocyanate method with an extraction kit (5 Prime → 3 Prime Inc, Boulder, CO, USA). Then, cDNAs were synthesized from HUVEC total RNA according to a manufacturer protocol (Perkin Elmer, Saint-Quentin-en-Yvelines, France). On the basis of the published cDNA sequence of DPI/II (Green et al., 1990), three oligonucleotides were synthetized (Fig. 1A) and used for PCR amplification and cDNA synthesis. PCR was run for 25 or 30 cycles (1 minute at 94°C as denaturation step; 2 minutes at 55°C as annealing step; 3 minutes at 72°C as elongation step). The PCR products were separated by agarose gel electrophoresis, and subcloned into the PCRII vector (TA cloning kit, Invitrogen, San Diego, CA, USA) for sequence analysis and further amplification.

cDNA sequencing was carried out according to the dideoxynucleotide chain termination method of Tabor and Richardson (1987) using a Sequenase DNA sequencing kit (United States Biochemicals, Cleveland, Ohio, USA).

Northern blot analysis

Samples (5 to 20 μg of total RNA per lane) were fractionated by standard formaldehyde/agarose gel electrophoresis and were transferred to Hybond-N filters (Amersham, Les Ulis, France). Hybridizations were performed using the cDNA fragments obtained by PCR amplification as ^{32}P-labelled probes as described (Golay et al., 1991).

Antibodies

The anti-DP antiserum (Ch-1) was a purified fraction obtained from a polyclonal antiserum raised against the synthetic peptide EKRRRKSVEDRFDQQKNDY corresponding to amino acids 2 to 20 from a partial DP sequence (Green et al., 1990). One rabbit was injected with 200 μg of the synthetic peptide coupled to keyhole limpet hemocyanin. It was boosted 5 times at two week intervals. Immunoaffinity purification of the anti-DP antiserum was carried out by loading a sample of the serum on a Sepharose 4B column (Pharmacia, Milwaukee, Wisconsin, USA) containing immobilized peptide. Specific antibodies were eluted with 0.1 M glycine (Sigma).

In addition, the following antibodies were used: mouse monoclonal

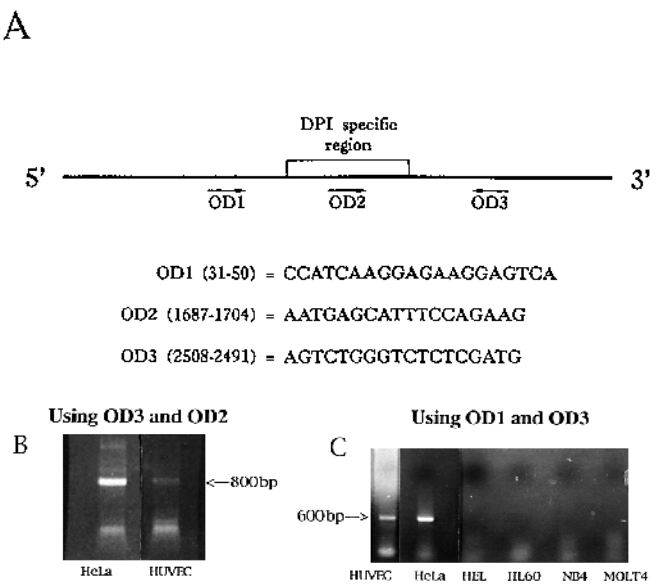

Fig. 1. (A) Human epithelial DP cDNA, position and sequence of primers. Base numbers are as for the partial sequence published by Green et al. (1990). (B) Gel electrophoresis of RT-PCR amplification products using primers OD3 and OD2: detection of a 800 bp fragment using RNAs from HeLa and HUVEC. (C) Gel electrophoresis of RT-PCR amplification products using primers OD3 and OD1: detection of a 600 bp fragment using RNAs from HUVEC and HeLa; no detectable band was observed using RNAs from hematopoietic cell lines HEL, HL60, NB4 and MOLT4.

antibody (mAb) to human VE-cadherin (clone TEA) (Leach et al., 1993) (Immunotech, Marseille, France); mAb to vimentin (clone V9, Sigma); mAb to β-catenin and p120 (Transduction Laboratories, Lexington, KY, USA); mAb to human PECAM-1 (clone 5F49, Hemeris, Grenoble, France); mAb to plakoglobin (clone PG5.1, Cowin et al., 1985; Franke et al., 1988); mAb to DPI/II (clones DP2-15, 2-17, Cowin et al., 1985) kindly provided by Prof. W. Franke, German Cancer Research Center, Heidelberg, Germany; polyclonal antiserum NW6 against a recombinant DPI/II C-terminal part corresponding to amino acids 1,151 to 1,730 (Angst et al., 1990), kindly donated by Dr K. Green, Northwestern University Medical School, Chicago, USA.

Preparation of protein samples and immunoblotting

Triton X-100 soluble cell fractions were prepared as described elsewhere (Lampugnani et al., 1995). Briefly, cells were extracted at 0°C with extraction buffer (10 mM Tris-HCl, 150 mM NaCl with 2 mM $CaCl_2$, pH 7.5, 1 mM PMSF, 40 U/ml aprotinin, 15 μg/ml leupeptin, 0.36 mM 1,10-phenanthroline, 1% Nonidet P-40 and 1% Triton X-100) for 30 minutes. The extraction buffer was collected and centrifuged at 15,000 ***g*** for 5 minutes. The supernatant was defined as the Triton X-100 soluble fraction.

Total cell fraction was obtained after incubation of the cells with Laemmli buffer (0.1 M Tris-HCl, pH 6.8, 25% β-mercaptoethanol, 10% SDS, 10% saccharose) for 5 minutes. The extract was collected and boiled for 5 minutes.

For immunoblotting, after SDS-electrophoresis, the polyacrylamide gel was incubated for 10 minutes in transfer buffer (50 mM Tris-HCl, 95 mM glycine, 10% methanol, 0.035% SDS). Separated proteins were then electrotransferred onto nitrocellulose. The blots were blocked with PBS containing 0.1% Tween (PBS-T) and 5% bovine serum albumin (fraction V, Sigma) (BSA) for 1 hour, incubated with Ch-1 at dilution 1:1,000 or rabbit pre-immune serum at dilution 1:1,000 or NW6 at dilution 1:10,000 (diluted in PBS-T with 2% BSA) and washed 3 times for 15 minutes in PBS-T. Then, the blots were incubated for 45 minutes with goat anti-rabbit IgG-peroxidase (Amersham), washed 3 times in PBS-T and revealed using ECL kit (Amersham).

Immunofluorescence microscopy

Cultures of HUVEC grown on fibronectin-coated coverslips were fixed in methanol at −20°C for 5-10 minutes then washed in PBS-T. Primary antibodies were applied for 30-45 minutes (Ch-1 and rabbit pre-immune serum at 1:10 dilution; NW6 at 1:50 dilution; mAbs at 5 μg/ml concentration), followed by 3 washes in PBS-T. Then, 1:100 dilution of secondary antibodies (affinity-purified goat anti-rabbit IgG or donkey anti-mouse IgG conjugated with fluorescein (FITC) or rhodamine (TRITC) both from Jackson, West Grove, Penn, USA) were incubated for 30 minutes, followed by 3 washes with PBS-T. For double staining, cells were incubated with anti-DP polyclonal antiserum (Ch-1) followed by FITC-coupled donkey anti-rabbit IgG. Then cells were exposed to the desired mAb followed by TRITC-coupled goat anti-mouse IgGs. Coverslips were mounted in Cytifluor

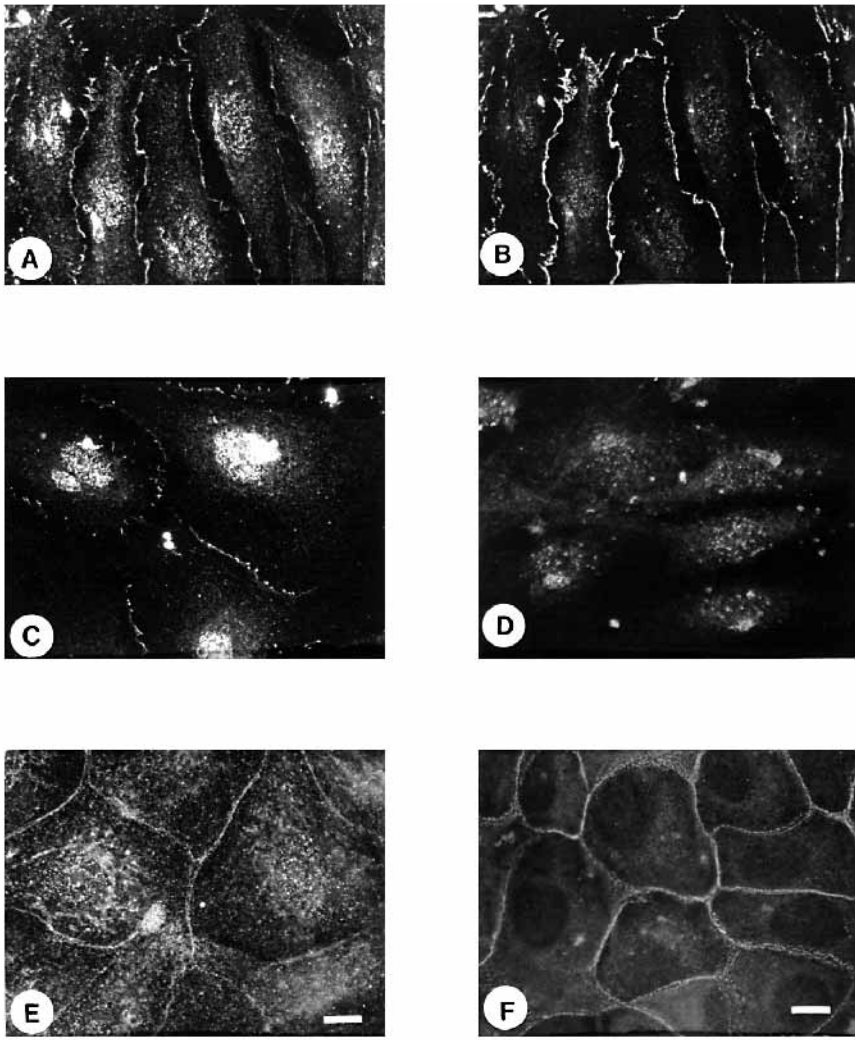

Fig. 2. Immunofluorescence microscopy of HUVEC (A-D) and HeLa (E-F) cell cultures. Double label immunolocalization of DP using polyclonal antiserum Ch-1 (A) and VE-cadherin using mAb TEA (B) showed that cell-to-cell contacts were positive for both DP and VE-cadherin. Immunofluorescence microscopy after the use of polyclonal antiserum NW6 to DP showed also a positive reaction at intercellular junctions (C). As a control, the rabbit pre-immune serum was negative at cell-to-cell contacts but retained the unspecific staining on the cell body (D). Immunolocalization of DP in HeLa using polyclonal antisera Ch-1 (E) and NW6 (F) showed a positive punctate reaction at cell-to-cell margins. Bars: (A-E) 10 μm; (F) 20 μm.

(Agar Scientific, Stansted, UK) and viewed by epifluorescence illumination using a Zeiss (Iena, Germany) microscope, and confocal microscopy.

Confocal microscopy

Series of optical sections were obtained with a confocal scanning laser microscope LSM410 (Zeiss). Fluorescence of FITC was obtained using the 488 nm excitation wavelength of the Argon laser and the emitted light was selected using a 510 nm dichroic filter and a 510-540 nm band-pass filter. Fluorescence of TRITC was obtained using the 514 nm excitation wavelength of the Argon laser and the emitted light was selected using a 560 nm dichroic filter and a long-pass filter with a cut-off wavelength of 590 nm. The optical sections were imaged with a ×63 oil immersion objective (NA 1.4, Planapochromat, Zeiss) and the measured resolution of the optical setting was 0.21 μm in the *x-y* plane and 0.45 μm along the *z* axis. The images of the FITC and TRITC fluorescence were recorded separately and the microscope stage was lifted up 0.5 μm between each optical section. The starting optical section (i.e. 0 μm) was defined at the level of contact between cell and coverslip. The gain and contrast of the photomultiplier detector were set in order to obtain an optimal imaging of the two types of fluorescence while limiting the crosstalk between channels.

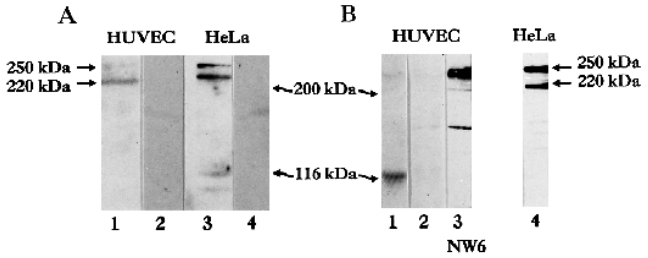

Fig. 4. Detection of DP expressed in HeLa and in HUVEC cell lysates by western immunoblotting. Cell extracts were separated by SDS electrophoresis, transferred onto nitrocellulose by western blotting and reacted with polyclonal antisera. (A) Immunoblotting using total cell extracts with Ch-1 (lanes 1 and 3) and with pre-immune serum (lanes 2 and 4). (B) Immunoblotting with NW6 using HUVEC subconfluent total cell fraction (lane 1) or Triton X-100 soluble fraction (lane 2) or confluent total cell fraction (lane 3) and HeLa total cell fraction (lane 4). Molecular mass markers are shown between A and B. Bands of the expected size (250 kDa for DPI and 220 kDa for DPII) were detected in total confluent cell extracts (A, lanes 1 and 3; B, lanes 3 and 4). These bands were not observed with pre-immune serum (A, lanes 2 and 4). In B, autoradiographs corresponding to lanes 1 and 2 were exposed for longer times than lanes 3 and 4 (lane 1, 6 minutes; lane 2, 4 minutes; lanes 3 and 4, 2 minutes). On lanes 1 and 2, a faint band at 250 kDa could be observed on the autoradiographs, corresponding to the size of DPI.

RESULTS

Localization of DP recognizing antibodies in HUVEC and HeLa cells

As shown in Fig. 2A and E, the polyclonal antiserum directed to a DP peptide (Ch-1, see Materials and Methods) decorated cell-to-cell margins both in HUVEC and in HeLa cells at confluence.

By conventional indirect immunofluorescence, double labelling of the cells with VE-cadherin and DP antibody showed a good codistribution of these two proteins in HUVEC (Fig. 2A,B). Diffuse background staining on the cell body was consistently observed even in HUVEC and HeLa cells when the antibody Ch-1 was used as immunopurified IgG.

Another polyclonal antiserum, NW6, presented a comparable type of localization at cell contacts and in the cell body of HUVEC and HeLa cells (Fig. 2C,F). This antibody was directed to a recombinant DPI/II C-terminal part (Angst et al., 1990) corresponding to a different DP region from the peptide used to produce Ch-1 antibody (see Materials and Methods).

In contrast, two mAbs, DP2.15 and DP2.17, to human DP were able to stain intercellular junctions in HeLa but did not give a detectable immunofluorescence signal in HUVEC (not shown).

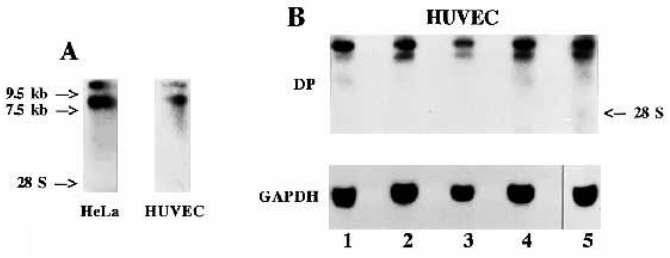

Fig. 3. (A) Northern blot analysis of total RNA from HeLa (5 μg) and confluent HUVEC (20 μg). Filters were exposed to autoradiography during 2 days for HeLa RNAs and 8 days for HUVEC RNAs. mRNA bands of the expected size (9.5 kb for DPI and 7.5 kb for DPII) were detected with the 800 bp probe.
(B) Northern blot analysis of total RNA (20 μg) from HUVEC either confluent (lane 1) or subconfluent (lane 2) or stimulated by LPS (lane 3), TGFβ1 (lane 4) and TPA (lane 5) (see Materials and Methods). DPI/II mRNA was detected using the 800 bp probe. To normalize RNA amounts, GAPDH mRNA was detected as a control.

Isolation and sequencing of partial DPI/II cDNAs in HUVEC

We then looked for the presence of DP mRNA in HUVEC. An RT-PCR method was first used. Three oligonucleotide sequences were chosen as primers on the basis of the published cDNA sequence of epithelial DP (Green et al., 1990). Oligonucleotides OD1 and OD3 are located in the common sequence of the two forms of DP (DPI/II), respectively, upstream and downstream of the region of splicing. OD2 is located within the spliced region (Fig. 1A).

Fig. 1B shows that, using HeLa and HUVEC RNAs, RT-PCR amplification with primers OD3 and OD2 gave the expected 800 bp cDNA fragment presumably corresponding to DPI sequence amplification. Using primers OD3 and OD1, a cDNA fragment 600 bp long (corresponding to the alternative spliced DPII sequence) was found (Fig. 1C). With these two oligonucleotides, another fragment of 2,100 bp, corresponding to the DPI cDNA fragment should be present. Apparently, this relatively long cDNA sequence was not effectively amplified with the RT-PCR conditions used in Fig. 1. Increasing the number of amplification cycles to 40 we could detect the band of 2,100 bp, but this was accompanied by a marked unspecific background (not shown).

As controls we used a series of human hematopoietic cell lines HEL, HL60, NB4, MOLT4 which were negative by RT-

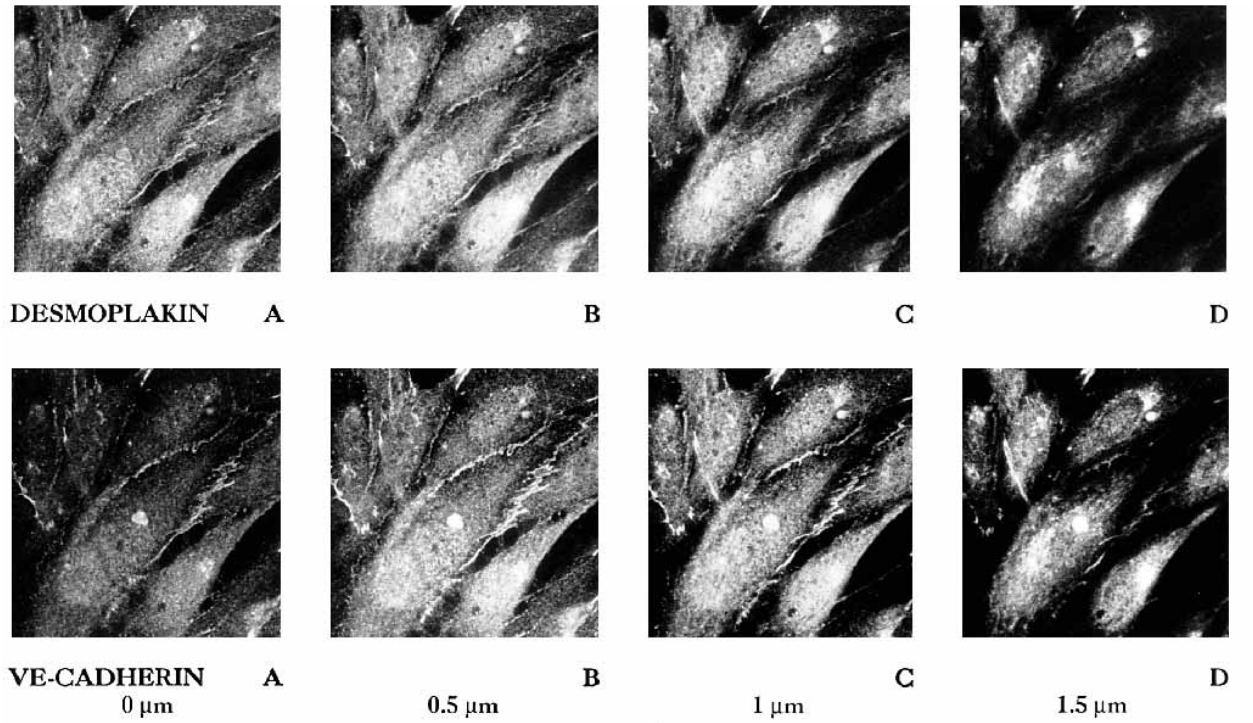

Fig. 5. Comparison of DP and VE-cadherin localization in confluent HUVEC using confocal microscopy. Cells were double stained with the polyclonal antiserum Ch-1 to DP and mAb TEA against VE-cadherin. The basal side of the cells is on the left (0 μm). The distance of the optical sections from this basal side is given in μm. DP labelling appeared to be equally intense at basal and intermediate levels (A and B), whereas VE-cadherin seemed to be concentrated at the intermediate plane (C). (D) DP was almost undetectable at subapical planes of the membrane. (A,B,C) VE-cadherin and DP were essentially colocalized. Bar, 10 μm.

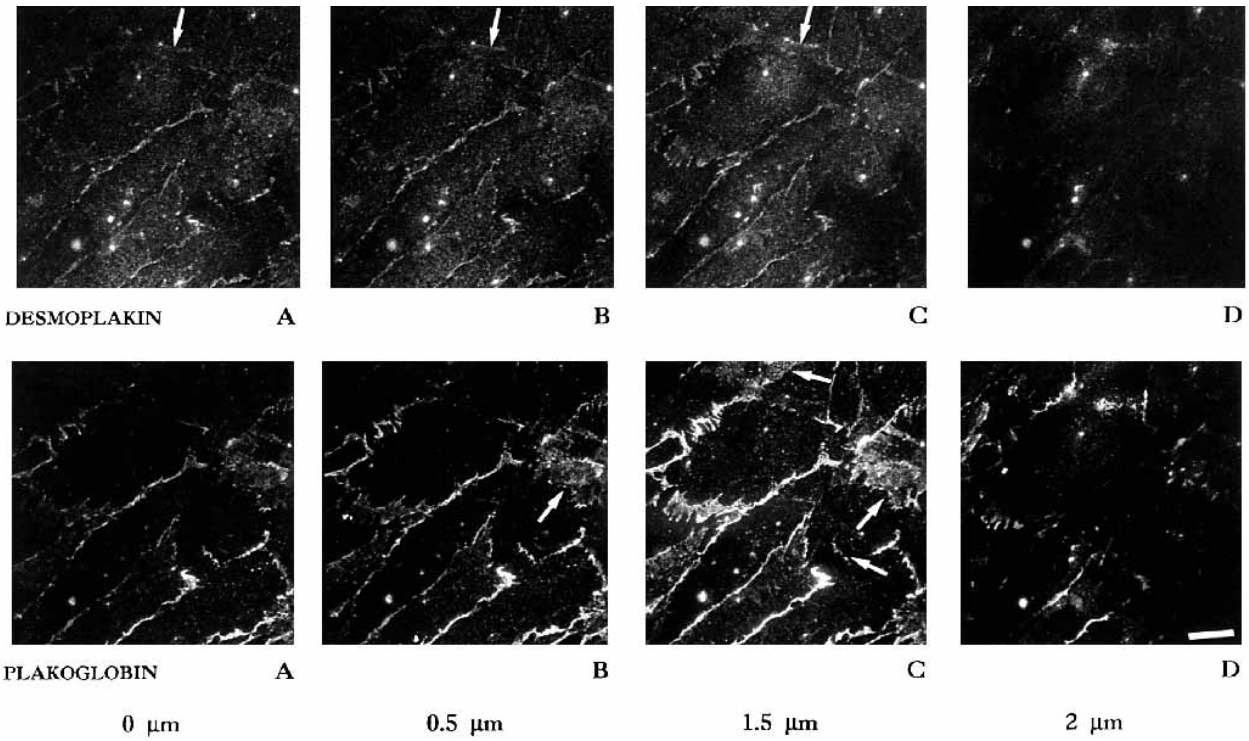

Fig. 6. Comparison of desmoplakin and plakoglobin localization in confluent HUVEC culture using confocal microscopy. Cells were double stained with polyclonal antiserum Ch-1 to desmoplakin and mAb PG5.1 against plakoglobin. The basal side of the cells is on the left. The distance of the optical sections from this basal side is given in μm. As observed in Fig. 5, DP labelling was equally distributed at basal and intermediate levels (A,B,C). Plakoglobin staining increased from basal (A) to intermediate levels (B,C). (D) At subapical sections of the membrane, plakoglobin was still present whereas DP essentially disappeared. (A,B,C) Arrows on upper photographs indicate regions with DP staining but devoid of plakoglobin staining and conversely arrows on lower photographs indicate regions with plakoglobin staining but devoid of DP staining. Bar, 10 μm.

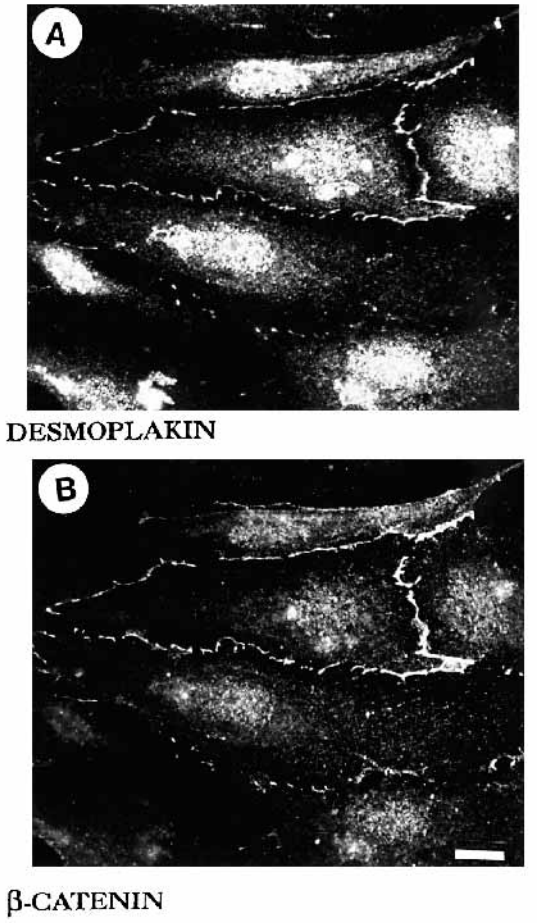

Fig. 7. Indirect immunofluorescence analysis of the codistribution of DP and β-catenin in confluent HUVEC. Cells were double stained for DP (A) using the polyclonal antiserum Ch-1 and β-catenin (B). Bar, 10 μm.

PCR analysis for DPI/II expression (Fig. 1C). Partial cDNAs of DPI (800 bp obtained with primers OD2 and OD3) and of DPII (600 bp obtained with primers OD3 and OD1) from HUVEC were cloned in PCRII vector and sequenced. The sequences of the 2 cDNA fragments were identical to the published sequences of epithelial DPI/II (not shown).

Northern blot analysis of DPI/II mRNA

The 800 bp and 600 bp cDNA fragments were amplified and used as probes for northern blot analysis of DPI/II mRNA in HUVEC. HeLa cells were used as positive control. Both probes identified two mRNAs of 9.5 and 7.5 kb in HUVEC and in HeLa cells (Fig. 3A). These mRNA sizes correspond to those previously reported for DPI and DPII in human and bovine tissues (Green et al., 1990). The expression of DPI/II was weaker in HUVEC than in HeLa cells, also considering that for this HUVEC cell type we seeded four times more RNA and exposed the filter for 8 days instead of 2 days. When normalized with the amount of RNA seeded per lane, the mRNAs of DPI and DPII expressed by HUVEC were not significantly modulated by cell confluency or by treatment with agents known to affect endothelial permeability (such as LPS or TPA) (Oliver, 1990; Klein et al., 1992; Yi and Ulich, 1992) or to increase the number of desmosomes in epithelial cells such a TGFβ1 (Yoshida et al., 1992) (Fig. 3B).

DP protein expression

To investigate whether the presence of DP mRNA correlated with protein expression we performed a western blot analysis of HUVEC and HeLa cell extracts. As reported in Fig. 4 two polyclonal antisera directed to different fragments of DP (Ch-1 and NW6) recognized two bands in HUVEC (Fig. 4A, lane 1, 4B, lane 3) and HeLa (Fig. 4A, lane 2, 4B, lane 4) total cell extracts corresponding to 250 and 220 kDa. The size of these bands corresponds to that of DPI and DPII in epithelial cells (Cowin et al., 1985). No detectable band corresponding to DP molecular mass was observed using a pre-immune rabbit serum (Fig. 4A, lanes 2 and 4). By western blot using HUVEC cell fractions, DPI/II was clearly detectable only in total extract of long confluent cells (Fig. 4B, lane 3). DPI was weakly detectable and DPII undetectable in total extract of subconfluent cells (Fig. 4B, lane 1) and in the Triton X-100 soluble fraction (Fig. 4B, lane 2). Bands between 120 and 200 kDa in Fig. 4B were not observed using pre-immune serum. They could correspond to degradation products produced during the manipulation of the cells as required by solubilization procedures. Lanes 1 and 3 in Fig. 4A show that using Ch-1 antibody the DPII band at 220 kDa was more visible than the DPI band at 250 kDa, whereas NW6 revealed equally DPI and DPII in HeLa extracts (Fig. 4B lane 4). This difference could be explained because the two antibodies are directed to different regions of the molecule and might have different affinities for the two DP forms.

Codistribution of DP with elements of adherence junctions

To further investigate the reciprocal spatial distribution of DP and of other junctional proteins, fixed and permeabilized HUVEC monolayers were double labelled and analyzed by confocal microscopy. As reported in Fig. 5, DP codistributed with VE-cadherin at different optical sections along the lateral membrane. According to the fluorescence settings we used (different laser wavelength, narrow bandwidth emission filters and separate imaging of the fluorescence channels) we can assume that the observed codistribution is not a consequence of a crosstalk between the two fluorescence emissions. Both molecules tended to be almost undetectable at subapical regions (Fig. 5D). A similar type of distribution was observed when DP staining was compared with that of plakoglobin (Fig. 6).

Plakoglobin and VE-cadherin were somehow more concentrated at the intermediate planes of the lateral cell membrane (Figs 5B,C and 6B,C). DP staining was equally intense at basal and intermediate focal planes. In a few regions, DP was absent in areas of plakoglobin staining and, vice versa, DP staining was not always accompanied by plakoglobin (Fig. 6A,B,C, see arrows). By conventional immunofluorescence, DP also codistributed with other members of adherence junctions such as β-catenin (Fig. 7) and p120 (not shown).

In other cell types DPI/II promotes intermediate filament anchorage in desmosomes (Schmidt et al., 1994). Endothelial cells do not have cytokeratins but they express vimentin (Schmelz et al., 1994). In confluent HUVEC vimentin staining was localized in an intense perinuclear aggregate from which

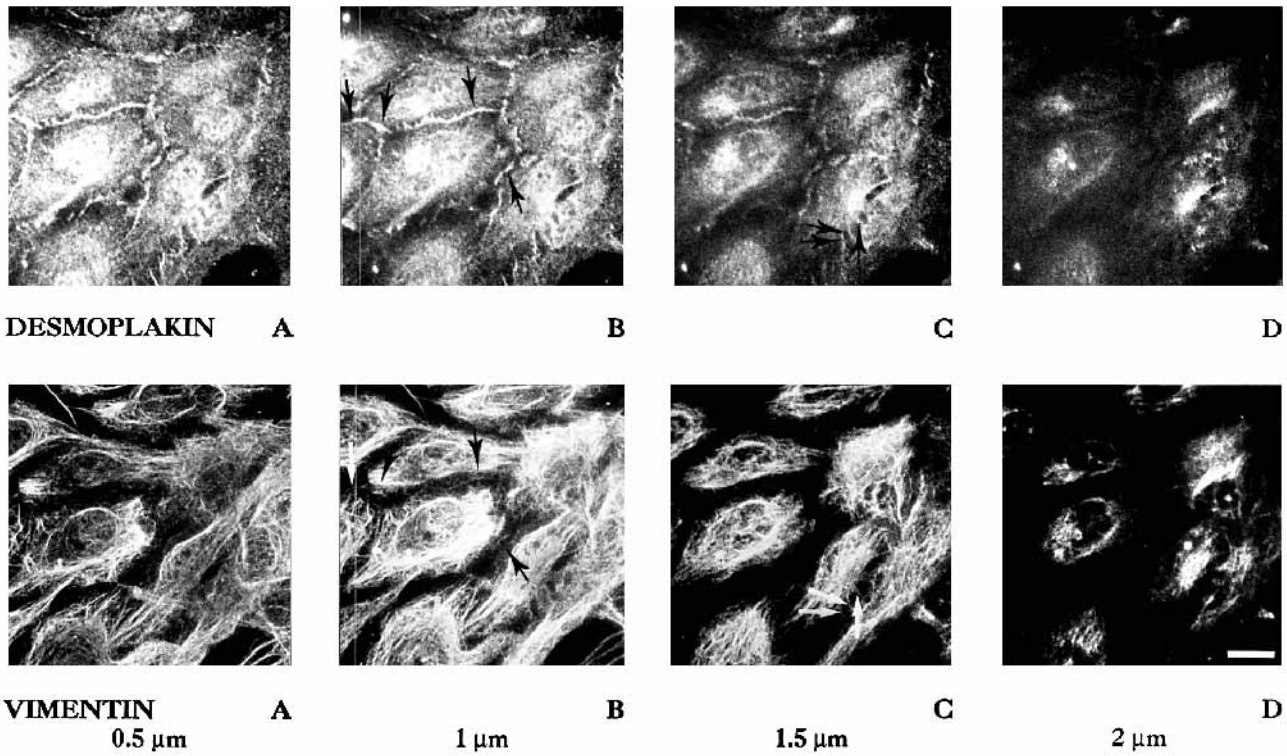

Fig. 8. Double immunofluorescence confocal microscopy of confluent HUVEC cultures with the polyclonal antiserum Ch-1 directed to DP and the mAb V9 to vimentin. The distance of the optical sections from the basal side of the cells is given in μm and starts 0.5 μm above the cell-coverslip contact. Intermediate filaments form a cytoplasmic network that stretches from the nucleus to the plasma membrane. Their density is highest in the subcortical cytoplasm near the nuclear envelope. However, regions where DP and vimentin were in correspondence could be found at cell-to-cell contacts (see black and white arrows). Bar, 10 μm.

a few bundles of filaments radiated toward the cell periphery (Fig. 8). By confocal microscopy vimentin filaments were concentrated at basal focal planes. Double labelling with DP and vimentin antibodies showed that DP sometimes could be found distributed with a few vimentin filament attachment sites at cell-to-cell contacts (Fig. 8, see arrows).

Another component of endothelial junctions is the immunoglobulin PECAM (Newman et al., 1990; Muller et al., 1992). PECAM is localized outside the AJ and does not codistribute with cadherins (Ayalon et al., 1994). As shown in Fig. 9, PECAM and DP were unrelated in the different focal planes of interendothelial clefts.

DISCUSSION

DPI/II has been commonly considered as a cytoplasmic marker of epithelial desmosomes (for review see Schmidt et al., 1994). By morphological criteria endothelial cells do not have structures comparable to desmosomes and do not express desmosomal components such as desmogleins, desmocollins or cytokeratins (Schmelz et al., 1993, 1994).

In this paper we report that HUVEC express DPI/II. The mRNA of this protein was detected both by RT-PCR and northern blot analysis. At the protein level, two antisera directed to different domains of DP recognized the molecule by western blot and indirect immunofluorescence staining.

Interestingly, two mAbs directed to bovine DP (DP 2.15 and DP 2.17) which recognize this molecule in a variety of cells (Cowin et al., 1985) did not give a detectable staining of HUVEC. This suggests that non lymphatic endothelial DP might have some specific feature. However, the molecular mass of endothelial DPI/II corresponds to that of DPI/II in epithelial cells and partial cDNA sequencing showed identity with the previously published sequence of the molecule. Another possible explanation for this discrepancy might be the limit of sensitivity of the fluorescence assay since DP expression in HUVEC is much lower than in epithelial cells. Alternatively DP may display different folding properties at non-desmosomal junctions in HUVEC and become less accessible to specific mAbs.

Through immunostaining of a large variety of tissues Schmelz et al. (1993, 1994) showed that DP was specifically present in lymph node retothelial cells and in lymphatic endothelia. These authors showed evidence for the codistribution of DP with VE-cadherin and suggested that these molecules might form specific junctional structures which correspond neither to desmosomes nor to adherence junctions. These structures were called ‘complexus adherentes’ in lymphatic vessels and ‘syndesmos’ in retothelial cells.

The data reported here confirm and extend these observations. The presence of DP in HUVEC suggests that this molecule might also play a more general role outside the lymphatic vasculature. Lymphatic vessels might, however, have a particularly high expression of DP as indicated by the positive staining with mAbs DP2.15 and DP2.17 which are negative on HUVEC (see above).

By the use of conventional indirect immunofluorescence and

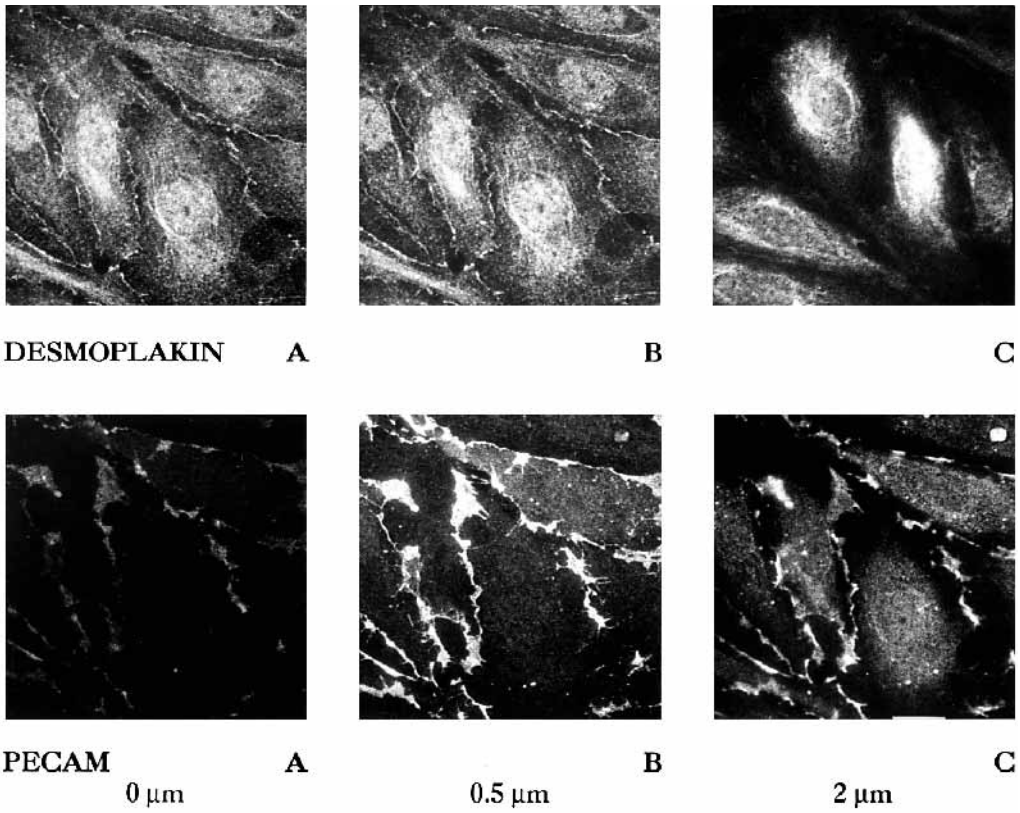

Fig. 9. Comparison of DP and PECAM localization in confluent HUVEC culture using confocal microscopy. Cells were double stained with the polyclonal antiserum Ch-1 to DP and the 5F49 mAb against PECAM. The basal side is on the left. The distance of the optical sections from the basal side is given in μm. The two proteins were present at the intercellular clefts, but their patterns of distribution were not superimposable. Bar, 10 μm.

confocal microscopy we found a good codistribution of DP with adherence junction components such as VE-cadherin, plakoglobin, β-catenin and p120. This type of DP distribution seems to be specific for AJ since DP did not colocalize with PECAM, a junctional protein concentrated outside of these structures (Muller et al., 1992; Ayalon et al., 1994).

Considering different optical sections of the endothelial clefts, DP was mostly concentrated at a basal/intermediate position along the lateral cell membrane. DP staining was in most of the cases associated with VE-cadherin or plakoglobin but in quite a few regions plakoglobin and DP did not codistribute. This suggests that plakoglobin and DP might form complexes where either protein is absent.

We report that DP protein expression becomes detectable only in long confluent cells. This is most likely due to post-transcriptional regulatory mechanisms since the DP mRNA level is not significantly modified by cell density (see Fig. 3B). Intracellular degradation of catenins and DP (Pasdar and Nelson, 1988; Kowalczyk et al., 1994) is strongly inhibited by their association with cadherins. In HUVEC, DP was absent in the detergent insoluble fraction both in subconfluent and confluent cells indicating that most of the detectable protein is indeed linked to the cytoskeleton.

In a few cases we observed that DP colocalizes at cell-to-cell contacts with vimentin microfilaments in a way somehow comparable to that described for desmosomes (Steinberg et al., 1987). This suggests that DP association with AJ components might drive vimentin microfilament anchorage.

Overall these data support the idea of Schmelz et al. (1993, 1994) of the presence of a specific type of intercellular junction in the endothelium. This type of junction (complexus adhaerentes) might be formed by VE-cadherin, catenins and DP eventually linked to vimentin.

DP usually coassembles with plakoglobin in desmosomes, in the endothelium we do not have elements to also exclude DP association with other catenins. Similarly, we do not know whether the complexus adhaerentes could be organized by the engagement of other cadherins different from VE-cadherin. However, to our knowledge, the major cadherins in HUVEC are VE- and N-cadherin (Rubin, 1992; Dejana et al., 1995). Since in most cases N-cadherin does not localize at cell-to-cell contacts in these cells (Salomon et al., 1992), VE-cadherin should play a major role in DP recruitment.

The biological significance of the complex interplay of cytoskeletal proteins at cadherin cytoplamic domain is still obscure. We have previously shown that endothelial AJ are dynamic structures and that the composition of the VE-cadherin/catenin complex might change at different phases of junction formation (Lampugnani et al., 1995). DP is detectable in the detergent insoluble fractions only in long confluent cells. It is therefore possible that this molecule gets associated with junctions at late stages of confluency and that promoting vimentin anchorage further strengthens cell connections and improves the control of endothelial permeability.

We thank Jerome Garin for peptide synthesis. F.B. was supported by AIRC (Associazione Italiana per la Ricerca sul Cancro) and by INSERM/ CNR exchange program.

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(Received 14 February 1996 – Accepted 7 May 1996)