Inhibition of cultured cell growth by vascular endothelial cadherin (cadherin-5/VE-cadherin).

L Caveda, … , M G Lampugnani, E Dejana

J Clin Invest. 1996;98(4):886-893. https://doi.org/10.1172/JCI118870.

Research Article

Endothelial cell proliferation is inhibited by the establishment of cell to cell contacts. Adhesive molecules at junctions could therefore play a role in transferring negative growth signals. The transmembrane protein VE-cadherin (vascular endothelial cadherin/cadherin-S) is selectively expressed at intercellular clefts in the endothelium. The intracellular domain interacts with cytoplasmic proteins called catenins that transmit the adhesion signal and contribute to the anchorage of the protein to the actin cytoskeleton. Transfection of VE-cadherin in both Chinese hamster ovary (CHO) and L929 cells confers inhibition of cell growth. Truncation of VE-cadherin cytoplasmic region, responsible for linking catenins, does not affect VE-cadherin adhesive properties but abolishes its effect on cell growth. Seeding human umbilical vein endothelial cells or VE-cadherin transfectants on a recombinant VE-cadherin amino-terminal fragment inhibited their proliferation. These data show that VE-cadherin homotypic engagement at junctions participates in density dependent inhibition of cell growth. This effect requires both the extracellular adhesive domain and the intracellular catenin binding region of the molecule.

Inhibition of Cultured Cell Growth by Vascular Endothelial Cadherin (Cadherin-5/VE-Cadherin)

L. Caveda,* I. Martìn-Padura,* P. Navarro,* F. Breviario,* M. Corada,* D. Gulino,‡ M.G. Lampugnani,* and E. Dejana*‡
**Istituto di Ricerche Farmacologiche Mario Negri, 20157 Milano, Italy; and ‡CEA, Laboratoire d'Hématologie, INSERM U 217, CEN-G, 38054 Grenoble, France*

Abstract

Endothelial cell proliferation is inhibited by the establishment of cell to cell contacts. Adhesive molecules at junctions could therefore play a role in transferring negative growth signals. The transmembrane protein VE-cadherin (vascular endothelial cadherin/cadherin-5) is selectively expressed at intercellular clefts in the endothelium. The intracellular domain interacts with cytoplasmic proteins called catenins that transmit the adhesion signal and contribute to the anchorage of the protein to the actin cytoskeleton. Transfection of VE-cadherin in both Chinese hamster ovary (CHO) and L929 cells confers inhibition of cell growth. Truncation of VE-cadherin cytoplasmic region, responsible for linking catenins, does not affect VE-cadherin adhesive properties but abolishes its effect on cell growth. Seeding human umbilical vein endothelial cells or VE-cadherin transfectants on a recombinant VE-cadherin amino-terminal fragment inhibited their proliferation. These data show that VE-cadherin homotypic engagement at junctions participates in density dependent inhibition of cell growth. This effect requires both the extracellular adhesive domain and the intracellular catenin binding region of the molecule. (*J. Clin. Invest.* 1996. 98:886–893.) Key words: endothelium • cadherins • cell proliferation • adherence junctions • catenins

Introduction

Endothelial cells constitute an important interface lining the internal vascular surface and regulating the passage of plasma proteins and circulating cells (1–3).

In vivo, intact endothelium presents a low turnover rate, however, when junctions are disrupted cells gain the capacity to migrate and proliferate. This capacity is then lost when cell to cell contacts are reorganized (4).

Endothelial cell junction components are therefore good candidates for transferring migration and growth inhibitory signals. Previous work (5) showed that protein membrane extracts from confluent endothelial cells were able to inhibit the growth of sparse endothelium but not of other cell types, suggesting the existence of membrane associated endothelial growth inhibitory proteins.

It was previously found that endothelial cells express a cell specific member of the cadherin family (cadherin-5 or vascular endothelial cadherin [VE-cadherin])[1] (6–8). This molecule is so far the only cadherin consistently organized at interendothelial adherence junctions (8–10). VE-cadherin is a constitutive component of all types of endothelia (8). As the other members of the family (11–15), VE-cadherin has adhesive properties and mediates homotypic cell adhesion (16). The intracellular domain interacts with cytoplasmic proteins called catenins (17) that transmit the adhesion signal and contribute to the anchorage of the protein to the actin cytoskeleton (18, 19).

In other types of tissues, cadherins can act as tumor suppressors. Reduced cadherin expression and/or activity is associated with enhanced tumor cell invasive potential and loss of differentiated characteristics (11–15, 18–23). In agreement with these observations, VE-cadherin expression was found to be strongly reduced in angiosarcomas (24).

In this paper we investigated the effect of VE-cadherin on cell growth. The results reported show that VE-cadherin can indeed transfer growth negative signals to the cells. This molecule can therefore contribute to density dependent inhibition of endothelial cell growth.

Methods

Cells. Human endothelial cells from umbilical vein (HUVEC) were isolated and cultured in M199 and 20% NCS (newborn calf serum) as previously described (8). Chinese hamster ovary (CHO) cells and mouse connective tissue fibroblast L929 cells were obtained from the American Tissue Type Collection and cultured in DMEM with 10% FCS (both from GIBCO, Life Technologies, Paisley, U.K.) (16). Full length VE-cadherin cDNA was cloned from human endothelial cells and inserted into pECE eukaryotic expression vector. CHO and L929 cells were cotransfected with pECE-VE-cadherin construct and pSV_2 neo plasmids by calcium phosphate precipitation as described (16). Control cells were transfected with empty pECE and pSV_2 neo plasmids, selected, cloned, and cultured as VE-cadherin transfectants (16). VE-cadherin expression in the clones used was comparable with that of HUVEC by Western and Northern blot analysis (16).

For transfection of CHO cells with truncated VE-cadherin, full length VE-cadherin cDNA cloned in pBluescript vector (16) was cut with the restriction enzyme Sma I (25). This digestion eliminated the

Address correspondence to E. Dejana, Istituto di Ricerche Farmacologiche Mario Negri, 20157 Milano, Italy. Phone: 2 39014480; FAX: 2 3546277.

Received for publication 13 December 1995 and accepted in revised form 28 May 1996.

J. Clin. Invest.

0021-9738/96/08/0886/08 $2.00
Volume 98, Number 4, August 1996, 886–893

1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; HUVEC, human umbilical vein endothelial cells; VE-cadherin, vascular endothelial cadherin.

coding region for the last 82 amino acids of the VE-cadherin cytoplasmic domain. CHO cells were transfected and selected as described (25). Transfectant clones expressing levels of truncated VE-cadherin comparable to wild type transfectants were used.

PECAM-1-L929 cells (26), integrin β1 CHO (27) and N-cadherin CHO (9) transfectants and respective controls were a kind gift of Dr. S. Albelda (University of Pennsylvania, Philadelphia, PA), Dr. G. Tarone (University of Torino, Torino, Italy) and Dr. B. Geiger, respectively. The expression of these molecules in each type of transfectant was comparable with that of HUVEC (9, 26, 27).

Antibodies and recombinant fragments. Mouse monoclonal antibodies (mAbs) to VE-cadherin were TEA1.31 (Immunotech, Marseille, France; 17), BV9 (Hemeris, Grenoble, France; 17) and BV6 (Bioline Diag., Giaveno, Italy; 17). The three mAbs recognized VE-cadherin extracellular domain. Using recombinant fragments and ELISA we found that TEA 1.31 and BV6 recognized epitopes in the region contained within amino acids 259-434, while BV9 recognized an epitope in the region contained within amino acids 1-268 (D. Gulino, unpublished observations). Rabbit polyclonal antibodies against α- and β-catenins were kindly provided by Dr. D. Vestweber (Max Planck Institute for Immunobiology, Freiburg, Germany; 17).

A recombinant VE-cadherin fragment (Cad-1), spanning the 546 amino acids of the extracellular domain from amino acid 1 to amino acid 434 was produced in *E.coli* as described previously (16, 28). The fragment was cloned into the expression vector pTG1924 containing the heat inducible promoter P_L and sequenced. As a negative control we used a fragment of the integrin GpIIb chain (28). Production and purification of the fragments was made as described (28).

Immunofluorescence microscopy. CHO cells were seeded on glass coverslips. At different times after seeding cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 2 min and processed for indirect immunofluorescence microscopy as described (25). Briefly, incubation with the primary antibody was followed by rhodamine-conjugated secondary antibody (Dakopatts, Glostrup, Denmark) in the presence of fluorescein-labeled phalloidin (2 μg/ml) with several washes with 0.1% BSA between the various steps. Coverslips were then mounted in Mowiol 4-88 (Calbiochem, La Jolla, CA). A Zeiss Axiophot microscope was used for observation and image recording on Kodak T max P3200 films.

Immunoprecipitation and immunoblot. To investigate the capacity of wild-type and truncated VE-cadherin to bind α- and β-catenins, cell extracts were immunoprecipitated with VE-cadherin mAb

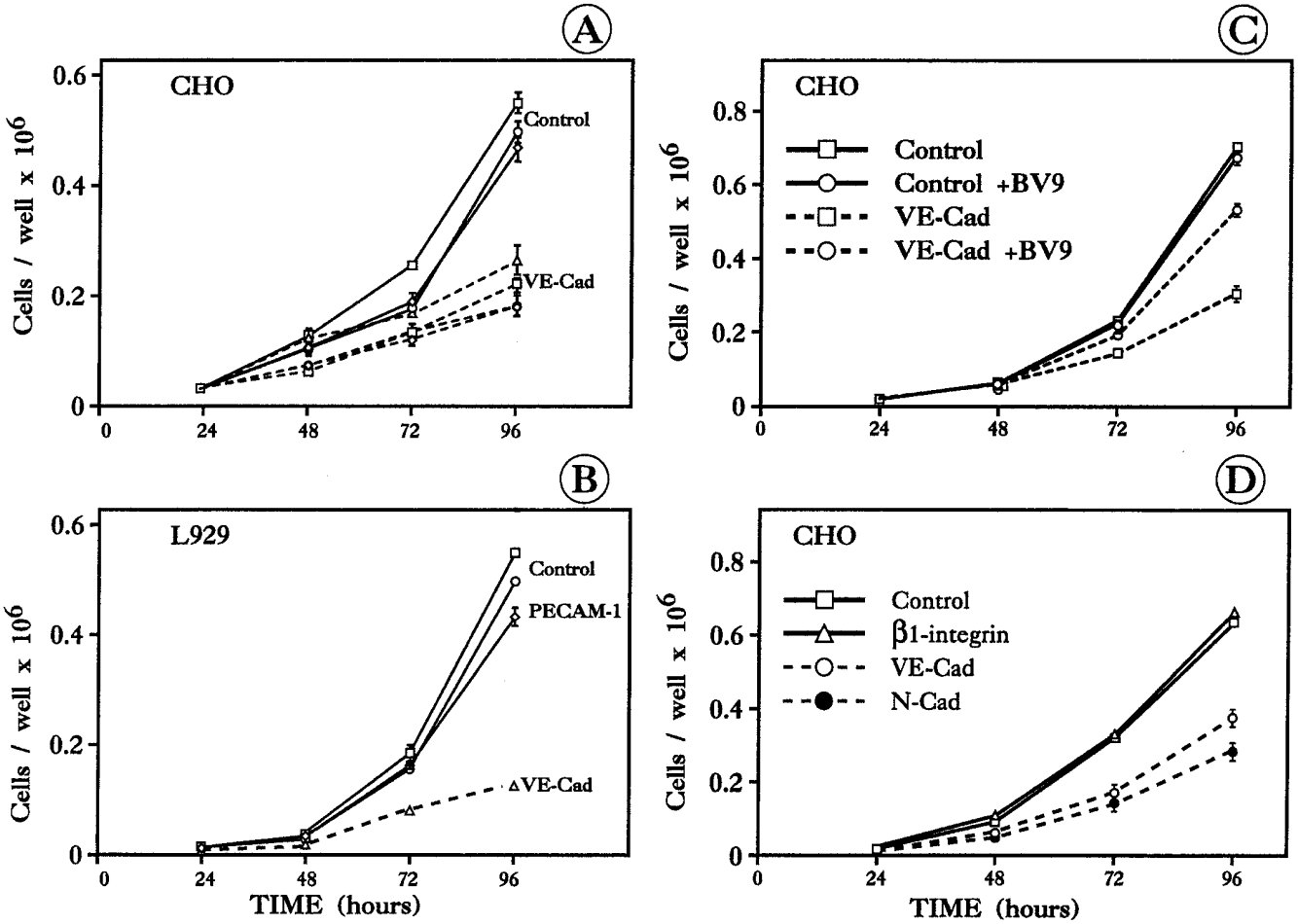

Figure 1. Effect of VE-cadherin transfection on cell growth. (*A*) Independent CHO cell clones transfected either with VE-cadherin (*dotted lines*, four clones) or empty vector (*continuous lines*, three clones) were tested for proliferation. Cells were detached and counted at different times after seeding. (*B*) Typical L929 cell clones transfected with VE-cadherin (--△--), PECAM-1 (--◇--), or empty vectors (--□--, --○--) were tested for proliferation as above. (*C*) Typical VE-cadherin or control CHO transfectants were seeded and cultured in the presence of an anti VE-cadherin mAb (BV9, 50 μg/ml, added daily) or an equal concentration of an irrelevant mouse IgG. The cells were tested for proliferation as above. (*D*) The growth of CHO cells transfected with the full length cDNA of integrin β1 (27) and N-cadherin (9) was measured in comparison with corresponding control and VE-cadherin transfectants. Values are means±SD of five replicates from a typical experiment out of at least four performed. SD bars do not appear in some points as they had dimension smaller than the symbols used.

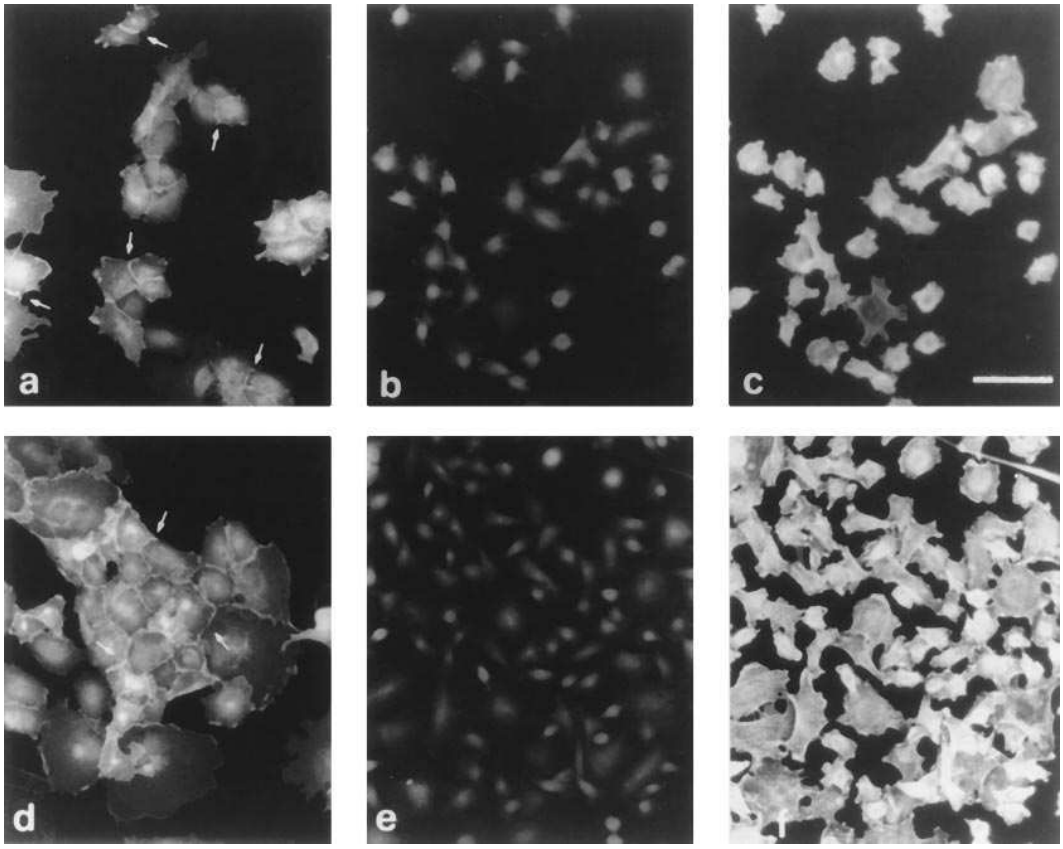

Figure 2. Morphological pattern of control and VE-cadherin transfected CHO cells. Cells were grown on glass coverslips for 30 h (*a–c*) and 48 hours (*d–f*). Cells were then fixed and immunostained with VE-cadherin mAb TEA 1.31 (*a, b, d,* and *e*). Control cells were double labeled with fluorescent-phalloidin for actin staining (*c* and *f*). VE-cadherin transfectants grow in small islands (*a* and *d*) while control transfectants tend to remain dispersed (*c* and *f*). VE-cadherin is localized at intercellular contacts (*arrows*) only in VE-cadherin transfectants (*a* and *d*) while no specific immunofluorescence signal was detected in control transfectants (*b* and *e*). Bar, 50 μm.

BV9 and then analyzed by immunoblot with VE-cadherin, α- and β-catenin Abs as described previously in detail (17, 25). Briefly, cells grown to confluence were extracted with 1% NP-40 in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 2 mM $CaCl_2$, 1 mM PMSF, 20 U/ml aprotinin, and 0.36 mM 1,10-phenanthroline for 20 min on ice. After scraping and centrifugation, cell extracts were precleared on protein G–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) and then incubated with protein G–Sepharose coupled to mAb BV9 for 2 h at 4°C. Immunocomplexes collected on the resin were washed and finally resuspended in Laemmli sample buffer boiling for 5 min. Samples were analyzed by SDS PAGE on 7.5% gel. The gel was then equilibrated in transfer buffer (50 mM Tris-HCl, 95 mM glycine, 1 mM $CaCl_2$) and electrotransferred onto nitrocellulose (Bio-Rad Laboratories, Richmond, CA). The membrane was blocked with 10% low fat milk in PBS with Ca^{2+} and Mg^{2+} and then incubated overnight at 4°C with the appropriate antibody at the optimal dilution in blocking buffer (1:2 dilution for TEA 1.31, BV9, or BV6 hybridoma culture supernatants and 10 μg/ml for rabbit purified IgG against α- or β-catenin). This was sequentially followed by 1 h of incubation at room temperature (RT) with peroxidase conjugated goat anti–mouse IgG (1 μg/ml) for mAbs or peroxidase conjugated protein A (1 μg/ml, Pierce Chemical Co., Rockford, IL) for rabbit polyclonal Abs. Peroxidase activity was revealed using an enhanced chemiluminiscence (ECL) kit (Amersham Int., Buckingham, UK).

Northern blot. For Northern blot analysis, RNA was extracted from cells and purified using the guanidium isothiocyanate/cesium chloride method as described (16). 15 μg of total RNA were fractionated on 1% agarose gel using formaldehyde-phosphate buffer, blotted onto nitrocellulose membrane and fixed at 80°C under vacuum for 2 h. Hybridization and washing conditions were as described (16, 29).

Cell growth assays. VE-cadherin is particularly sensitive to trypsin in absence of Ca^{2+}. Cells were detached in a way to maximally preserve VE-cadherin at the cell surface, as described previously in detail (16). Briefly, 0.01% trypsin (from bovine pancreas, type III; Sigma Chemical Co., St. Louis, MO) in Hank's balanced salt solution with 25 mM Hepes (HHBSS), 10 mM $CaCl_2$ and 5 mM $MgCl_2$, was added and maintained on the cells for the shortest interval after which initial intercellular retraction appeared (usually 5–7 min, the progression of trypsin effect was carefully monitored under microscope). Cells were completely and rapidly detached by vigorously shaking the flasks. Trypsin action was stopped adding DMEM with 10% FCS and 0.1% soybean trypsin inhibitor (SBTI; Sigma Chemical Co.). Cells were then centrifuged and resuspended in complete medium for plating. By FACS® analysis about 85–90% of surface VE-cadherin was detectable after this procedure. In some experiments (see Fig. 5 *B*) VE-cadherin was digested by detaching the cells with trypsin (O.O2%) in the presence of EDTA (O.5 mM) as described (8). Trypsin action was then blocked and cells treated as above. In these conditions, by FACS® analysis, VE-cadherin expression on the cell surface was below 10%.

For the growth assay, cells were plated at 1×10^4/ml (1 ml/well) in 24-well plates (2 cm^2 /well). Culture medium was not changed for the duration of the experiment (96 h). Cell number was evaluated by counting the cells (five replicates) after trypsinization. mAb BV9 (50 μg/ml) was added daily. As negative controls irrelevant mouse IgG or two other VE-cadherin mAbs, TEA 1.31 and BV6 (16, 17), with no activity in this assay up to 100 μg/ml were used.

To test the effect of recombinant fragments on cell growth, 96-well plates were coated with different concentrations of the fragments or 1% BSA in PBS for 1h at 37°C and then with 1% BSA for another hour at 37°C. Cells were seeded at 5×10^3/ml (200 μl/well) and cultured for 96 h in DMEM with 10% (CHO) and 20% (HUVEC) FCS without changing medium. Cells were fixed, stained with crystal violet dye (Diff-Quick, Baxter Dade AG, Dudingen, Switzerland) and counted measuring adsorbance at 540 nm with an Automated Microplate Reader (30).

To evaluate DNA synthesis a double staining of total nuclei (Hoechst 33342) and nuclei of cells in S phase was performed. CHO

cells were cultured on glass coverslips in 24-well plates (as above). 50 μM bromodeoxiuridine (BrdU) was added to the culture medium for 6 h at 37°C after which cells were washed with PBS and fixed in 3% paraformaldehyde at RT for 20 min. Coverslips were then washed twice with PBS and incubated with 0.1 M glycine in PBS to quench residual paraformaldehyde. Permeabilization was performed with 0.1% TX-100 in PBS for 4 min at 4°C. BrdU was revealed by anti-BrdU mAb (IgG2a, Amersham) followed by goat anti-mouse IgG2a TRITC-conjugated antibody (31). Cell nuclei were stained with Hoechst 33342 (2 μg/ml in PBS) for 2 min followed by three washes in PBS (31). Coverslips were then mounted and examined with a Zeiss Axiophot microscope. The relative inhibition of DNA synthesis was calculated using the formula: = [% BrdU positive cells (control transfectant) − % of BrdU positive cells (VE-cadherin transfectant)] / % BrdU positive cells (control transfectant).

Cell aggregation. To measure calcium-dependent aggregation cells were detached by trypsin in the presence of Ca^{2+} as described above. Cells were then washed twice and resuspended at a density of 4×10^5 cells/ml in HHBSS with 1% BSA. Cell suspension (0.5 ml/well) was seeded in a 24-well plate previously coated with 1% BSA and allowed to aggregate in the presence of 5 mM $CaCl_2$ or 5 mM EGTA for 90 min at 37°C on a rotating platform (80 rpm). The reaction was stopped by addition of 5% glutaraldehyde in PBS (0.5 ml/well). The initial number of particles (Nt_0) and the number of particles at 90 min (Nt_{90}) were counted using a ZM Coulter Counter. Quantification of aggregation was estimated by the index $[(Nt_0 - Nt_{90})/Nt_0] \times 100$ (16, 32).

Results

Effect of VE-cadherin transfection on cell growth. To study the effects of VE-cadherin on cell growth we first transfected human VE-cadherin cDNA in two cell lines (CHO and L929 cells) that did not present contact inhibition of growth and which do not express cadherins (16, 33). The level of expression of VE-cadherin in the transfectant clones was validated by Northern and Western blot and by immunofluorescence microscopy, as described previously in detail (16). The VE-cadherin transfected clones showed comparable levels of both VE-cadherin protein and mRNA with respect to the endogenous endothelial gene (16). In addition, when adherent monolayers were studied by immunofluorescence microscopy after staining with VE-cadherin mAbs, both VE-cadherin transfectants and endothelial cells appeared 100% positive. In both cell types the antigen showed a selective localization at cell to cell junctions (reference 16 and see Fig. 2).

As shown in Fig. 1 *A*, transfection of VE-cadherin induced inhibition of cell growth in all the independent CHO transfectant clones analyzed. Similarly, transfection of VE-cadherin in L929 cells inhibited cell proliferation (Fig. 1 *B*). A significant inhibition of cell growth was noticed even at relatively low cell density. This can be explained by the fact that VE-cadherin transfectants grow in small islands and cell to cell contacts are established and maintained immediately after cell division (Fig. 2).

Addition of VE-cadherin mAb (BV9) recognizing an epitope on the extracellular domain of the molecule (reference 17 and Methods) partially reversed growth inhibition of VE-cadherin transfectants (Fig. 1 *C*). As negative controls either irrelevant mouse IgG (Fig. 1 *C*) or two mAbs recognizing VE-cadherin (BV6 and TEA 1.31) but inactive in this assay (not shown), were used.

BV9 (50 μg/ml, added daily) had a small but consistent effect on HUVEC cell growth. BV9 induced 15–20% increase in cell number (range of four experiments) in comparison to the same concentration of an inactive mAb, at 96 h after cell seeding. No significative inhibition was detected at shorter times after cell seeding or at higher concentrations of the mAb (data not shown).

A

Transfectants	48 HOURS % Bdr U positive cells	48 HOURS Relative inhibition	72 HOURS % Bdr U positive cells	72 HOURS Relative inhibition
Control	36 ± 2	-	53.5 ± 2	-
VE-Cadherin	28.1 ± 1	23	30.4 ± 3	44

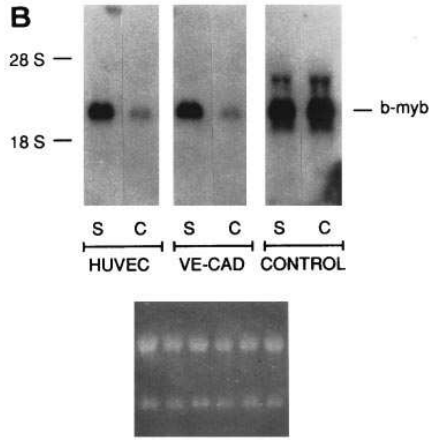

Figure 3. Effect of VE-cadherin transfection on DNA synthesis and B myb expression. (*A*) VE-cadherin and control CHO transfectants were cultured on glass coverslips in 24-well plates. To evaluate DNA synthesis double staining of total nuclei (Hoechst 33342) and nuclei of cells in S phase (anti BrdU) was performed. The relative inhibition of DNA synthesis was calculated with the formula: = [% BrdU positive cells (control transfectant) − % BrdU positive cells (VE-cadherin transfectant)]/% BrdU positive cells (control transfectant). The data are means±SD of 15 fields of 1.2 mm^2. (*B*) Northern blot analysis of B myb expression in VE-cadherin and control CHO transfectants and in HUVEC as a function of cell density. *S*, sparse cells (6×10^4 cells/cm^2 for both types of transfectants, and 14×10^3/cm^2 for HUVEC); *C*, confluent cells (25×10^4 cells/cm^2 for VE-cadherin transfectants; 50×10^4 cells/cm^2 for control transfectants and 60×10^3 cells/cm^2 for HUVEC). Equal amounts of RNA were seeded as shown by ethidium bromide staining (*lower panel*).

Transfection of other transmembrane proteins enriched at cell contacts as PECAM-1 (34) (Fig. 1 *B*) and integrin β1 chain (35) (Fig. 1 *D*) caused very little if any inhibition of cell growth. In contrast, transfection of another cadherin (N-cadherin) (9) inhibited cell proliferation in a way comparable with that induced by VE-cadherin (Fig. 1 *D*).

We tested whether VE-cadherin transfection inhibited DNA synthesis. As reported in Fig. 3 *A*, BrdU positive nuclei were less frequent in VE-cadherin than in control transfectants. B-myb, a transcriptional factor required for cell growth (36), was also downregulated by cell confluence in HUVEC and in VE-cadherin transfectants but not in control transfectants (Fig. 3 *B*).

Effect of VE-cadherin binding to catenins. The results described above suggest that VE-cadherin expression can transfer negative growth signals to the cells. To get some insight on VE-cadherin mediated signalling we next studied whether association of the molecule with catenins was required. To this purpose we transfected CHO cells with a cytoplasmic truncated mutant of VE-cadherin as described in detail elsewhere

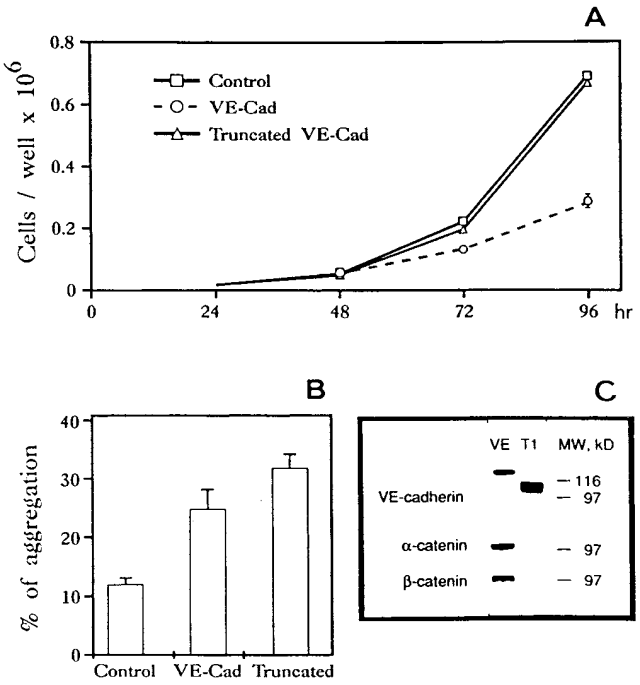

Figure 4. VE-cadherin binding to catenins and inhibition of cell growth. (*A*) Transfection of a VE-cadherin mutant truncated in the cytoplasmic domain did not affect cell growth. The figure shows the proliferation of CHO cells transfected with wild type VE-cadherin, truncated mutant VE-cadherin or control plasmid. Values are means of five replicates from a typical experiment out of three performed. SD bars do not appear in some points as they had a dimension smaller than the symbols used. (*B*) Truncated VE-cadherin retained aggregating properties. The figure shows the values of homotypic Ca^{2+} dependent cell aggregation of wild type VE-cadherin, truncated mutant VE-cadherin and control transfectants. Cell aggregation was evaluated as described in Methods. The values are means±SD of four separate experiments. (*C*) Truncated VE-cadherin was unable to bind α- and β-catenins. Cell extracts from wild type (VE) or truncated mutant (T1) VE-cadherin transfectants were immunoprecipitated with VE-cadherin mAb BV9 and then analyzed by immunoblot with VE-cadherin, α- and β-catenin antibodies as described (Methods and reference 25). As expected, truncated VE-cadherin runs with an apparent molecular weight lower than the wild type molecule. By Western blot two intense bands were detected in the truncated VE-cadherin transfectants one of 110 kD corresponding to the expected size and one of 98 kD that corresponds to an immature intracellular form of the mutant VE-cadherin (see reference 25 for details). Surface expression of the wild type and truncated forms of VE-cadherin cDNA was comparable (25). Wild-type transfected VE-cadherin was associated with two major polypeptides with apparent molecular masses of 105 and 95 kD recognized by α- and β-catenin antibodies respectively (lane *VE*). These polypeptides were absent in immunoprecipitates from truncated VE-cadherin transfectants (lane *T1*). To save antibody and directly compare the level of VE-cadherin and catenins in the same sample, after blotting the nitrocellulose was cut perpendicularly to the direction of protein run, separating the areas of VE-cadherin and each catenin band. This was done with the reference of molecular weight standards. The sheets were then reacted with the appropriate antibody to reveal VE-cadherin or associated catenins as described in detail (17, 25). The migration of molecular weight markers is shown on the right.

(25). The mutant lacks the cytoplasmic domain spanning amino acids 702 to 784 which is responsible for catenin association (37, 38).

The level of expression of truncated VE-cadherin in transfectant clones was comparable with that of the wild-type molecule (reference 25 and Fig. 4).

As expected, by immunoprecitation and immunoblot analysis, truncated VE-cadherin was unable to link α- and β-catenins (reference 25 and Fig. 4 *C*). Detection of other catenins (more specifically plakoglobin) could not be done for the lack of hamster cross reacting antibodies. However, the region of linkage of plakoglobin to cadherins corresponds to the deleted sequence (37, 38). It is therefore very likely that this catenin too cannot bind to the truncated mutant.

In contrast to previously described cadherins (37, 38), truncated VE-cadherin retained Ca^{2+} dependent aggregating prop-

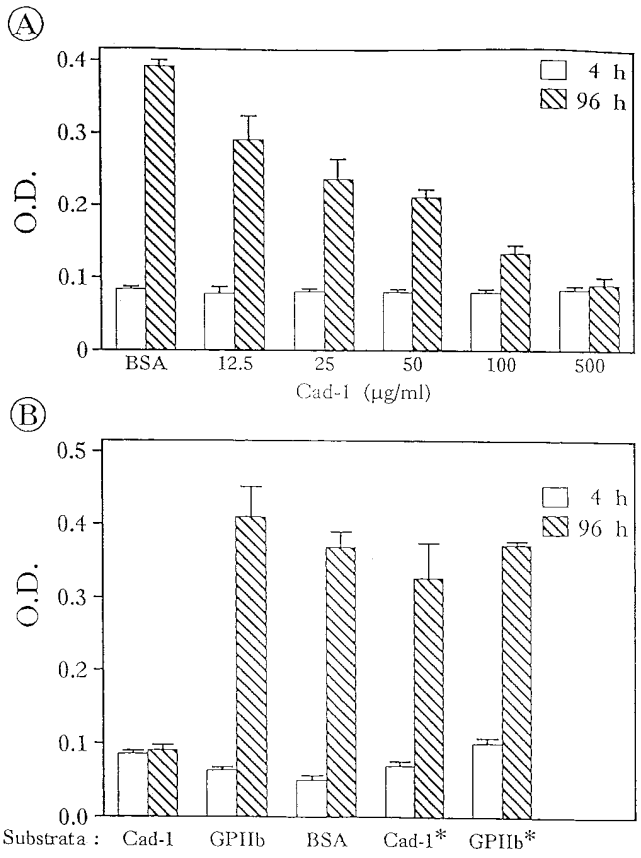

Figure 5. Immobilized VE-cadherin recombinant amino-terminal fragment inhibits endothelial cell growth. (*A*) Culture 96-well plates were coated with different concentrations of VE-cadherin fragment (Cad-1) or 1% BSA (BSA) as described in Methods. HUVEC were fixed and stained 4 and 96 h after seeding on different concentrations of VE-cadherin recombinant fragment. Data are means±SD of four replicates from a typical experiment out of three performed. (*B*) Culture 96-well plates were coated with VE-cadherin (Cad-1), integrin GpIIb (GpIIb) fragments (200 μg/ml), or 1% BSA (BSA). HUVEC were detached by trypsin treatment either in the presence (columns Cad-1, GpIIb, and BSA) or absence (columns Cad-1* and GpIIb*) of Ca^{2+} in order to preserve or digest VE-cadherin respectively. Cells were fixed and stained 4 h and 96 h after seeding on the different substrata. Data are means±SD of four replicates from a typical experiment out of three performed.

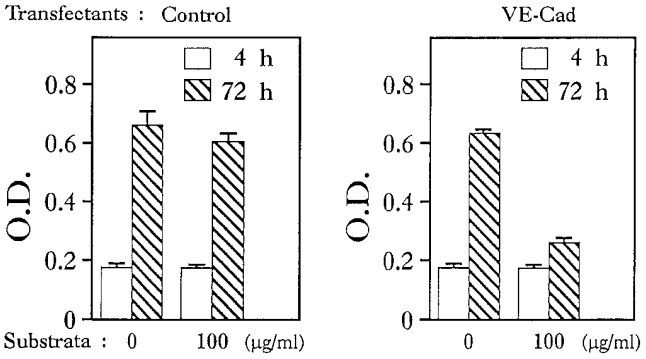

Figure 6. Immobilized VE-cadherin recombinant amino-terminal fragment inhibits the growth of VE-cadherin but not control transfectants. Control and VE-cadherin CHO transfectants were seeded on immobilized VE-cadherin recombinant fragment (Cad-1). Cells were fixed and stained at 4 and 72 h as above. Data are means±SD of four replicates from a typical experiment out of three performed.

erties (reference 25 and Fig. 4 *B*) and concentrated at cell to cell contacts as well as the wild type form (25). Therefore, deletion of the cytoplasmic tail did not affect the adhesive properties of the protein, however, as shown in Fig. 4 *A*, it abolished its growth inhibitory activity.

Effect of VE-cadherin recombinant fragments on endothelial cell growth. To evaluate whether the engagement of VE-cadherin could inhibit HUVEC growth, we tried to mimic confluence by seeding the cells on an immobilized VE-cadherin recombinant fragment.

Seeding HUVEC on a VE-cadherin fragment comprising amino acids 1 to 434 out of the 546 of the extracellular domain caused a concentration dependent inhibition of cell proliferation at 96 h (Fig. 5 *A*). This was not due to an inhibitory effect of the fragment on cell adhesion since the number of cells adherent at 4 h was unaffected.

The effect of VE-cadherin fragment on cell growth seems to be specific since: (*a*) seeding HUVEC on an irrelevant recombinant fragment (GpIIb) did not alter cell growth up to 96 h (Fig. 5 *B*); (*b*) when CHO transfectants were seeded on VE-cadherin fragment, only cells expressing VE-cadherin but not control transfectants had a reduced growth (Fig. 6); (*c*) when HUVEC were detached by trypsin and EDTA to destroy VE-cadherin (Methods and reference 8) the inhibitory effect of the VE-cadherin fragment could not be observed (Fig. 5 *B*).

This last point seems surprising since by flow cytometry analysis VE-cadherin recovery after cell treatment with trypsin and EDTA was complete at 24 h (data not shown). However we have previously shown (39) that HUVEC release and organize a complex matrix of endogenous proteins such as fibronectin and von Willebrand factor within 2 h after seeding. If VE-cadherin cannot be engaged at the moment of cell seeding (as in cells treated with trypsin and EDTA), it is difficult for the neosynthetized molecule to have full access to VE-cadherin recombinant fragment.

Discussion

We report here that transfection of VE-cadherin cDNA inhibits cell proliferation. This effect was apparent in different transfectant clones and in two separated cell lines (CHO and L929). Growth inhibition was not related to a general effect of transfection of cell surface proteins since transfection of PE-CAM-1, β1 integrin chain and more specifically of the truncated mutant of VE-cadherin did not affect cell proliferation. In contrast, the transfer of N-cadherin cDNA was as effective as VE-cadherin in inhibiting cell growth. This suggests that this effect might be shared by different members of the cadherin family.

N-cadherin is expressed by endothelial cells but, in contrast to transfectants, is not localized at junctions (9). This leaves open the question whether it can actually contribute to density dependent inhibition of growth in this cell type.

It is conceivable that VE-cadherin exerts its inhibitory role when engaged and clustered at cell contacts (16, 17). The data presented here are consistent with this hypothesis. The effect of VE-cadherin expression was more marked at higher cell density (for instance in Fig. 1, compare 96 to 72 h after plating). In addition, the direct engagement of VE-cadherin induced by seeding HUVEC or VE-cadherin transfectants on VE-cadherin recombinant fragment caused inhibition of cell growth. This suggests that the effect of VE-cadherin can be mediated by the homotypic binding and immobilization of the molecule.

Interestingly, it was found (A.S. Yap and B.M. Gumbiner, unpublished data) that seeding C-cadherin transfectants on C-cadherin recombinant fragments causes clustering of the cadherin and α- and β-catenins. This supports the idea that even when cadherins are engaged at the basal aspect of the cell membrane they can trigger the assembly of "adherence junction-like" structures.

Trypsin and EDTA treatment of HUVEC abolished the growth inhibitory effect of VE-cadherin recombinant fragment. By flow cytometry analysis this treatment digests most of VE-cadherin on the cell surface, but full recovery is obtained within 24 h (not shown). The fact that no inhibition of growth is observed up to 96 h can be explained by the observation that endothelial cells release and organize a complex matrix of endogenous proteins such as fibronectin and von Willebrand factor within few hours after seeding (39). This matrix would cover the recombinant fragment. To transfer growth negative signal, it seems therefore important that VE-cadherin could be engaged at the moment of cell seeding.

An important question is related to the mechanism of action of VE-cadherin. The tumor suppressor activity of classic cadherins has been related to their adhesive properties, i.e., inhibiting cell detachment from primary tumors would inhibit invasion and metastatic dissemination (11–15, 18–23). VE-cadherin seems to exert a direct activity on cell proliferation. The truncated VE-cadherin form, while retaining adhesive properties, has lost the capacity to inhibit cell growth. This suggests that VE-cadherin could transfer growth inhibitory signals through association with catenins or other cytoplasmic molecules.

It has been recently found that α- and β-catenins bind to the tumor suppressor gene product adenomatous polyposis coli (APC) (40, 41). APC mutations in the region responsible for binding to catenins are associated with the development of hyperplasia, an early event in tumorigenesis (40, 41).

As recently suggested (42), a possible model of signal transduction through cell junctions is that cadherins, when recruited at cell to cell contacts, sequester catenins from an intracellular pool reducing the amount of these molecules available for linking signal transduction components. Interestingly, the pro-

tooncogene Wnt-1 causes upregulation of β-catenin and stabilizes its binding to cadherins (43). While this manuscript was in preparation, Watanabe et al. (44) reported that transfection of PC9 tumor cells with α-catenin in order to activate E-cadherin caused retardation of cell growth.

All these data are consistent with the fact that cadherins and catenins can directly contribute to inhibition of cell growth induced by cell density. Surprisingly, this phenomenon was not evidenced earlier. It might be that growth inhibition by cadherins present different intensity as a function of cell and cadherin type. For instance, tumor cell lines used for transfection might express altered amounts of catenins (44) which might be the cause for altered signalling capacity by cadherins (see also reference 45).

Inhibition of B-myb expression in both confluent HUVEC and VE-cadherin transfectants, but not in control transfectants, suggests that this gene, which is required for cell proliferation, is directly or indirectly downregulated by cell density and more specifically by VE-cadherin engagement. Studies are under way to define at which point of the cell cycle the establishment of cell to cell contacts and more specifically VE-cadherin can act.

Endothelial cell junctions are very complex organelles formed by different transmembrane and cytoplasmic components (10). Besides VE-cadherin, it is likely that other molecules can contribute to control contact dependent growth inhibition. It was shown that seeding of endothelial cells on PECAM-1 recombinant fragments could inhibit their growth (46). In this report, however, PECAM-1 transfection shows a very poor inhibition of cell proliferation. More detailed studies are required to clarify the reciprocal role of these molecules.

Even if the observations reported using HUVEC remain to be substantiated in situ in the tumor microvasculature, they might open a new direction in the development of agents able to modulate endothelial cell growth.

Acknowledgments

This work was supported by the Italian National Research Council (Special Project "Applicazioni Cliniche della Ricerca Oncologica"), by the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), European Community Biomed 2 (contracts: PL95O669 and BMH4CT950875). F. Breviario is partially supported by NATO grant CRG941228. P. Navarro is a recipient of a "Human Capital Mobility" fellowship from EEC. L. Caveda is on leave of absence from Center of Pharmaceutical Chemistry, Havana, Cuba.

References

1. Gimbrone, M.A. 1986. Vascular endothelium: Nature's blood container. *In* Vascular Endothelium in Hemostasis and Thrombosis. M.A. Gimbrone, Jr., editor. Churchill Livingstone, Edimburgh, UK.1–24.

2. Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem.* 267:10931–10934.

3. Ross, R. 1986. The pathogenesis of artherosclerosis — an update. *N. Engl. J. Med.* 314:488–500.

4. Schwartz, S.M., C.M. Gajdusek, and S.C. Selden. 1981. Vascular wall growth control: the role of the endothelium. *Artherosclerosis.* 1:107–126.

5. Heimark, R.L., and S.M. Schwartz. 1985. The role of the membrane-membrane interactions in the regulation of endothelial cell growth. *J. Cell Biol.* 100:1934–1940.

6. Suzuki, S., K. Sano, and H. Tanihara. 1991. Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. *Cell Regulation* 2:261–270.

7. Heimark, R.L., M. Degner, and S.M. Schwartz. 1990. Identification of a Ca^{2+}-dependent cell-cell adhesion molecule in endothelial cells. *J. Cell Biol.* 110:1745–1756.

8. Lampugnani, M.G., M. Resnati, M. Raiteri, R. Pigott, A. Pisacane, G. Houen, L.P. Ruco, and E. Dejana. 1992. A novel endothelial specific membrane protein is a marker of cell–cell contacts. *J. Cell Biol.* 118:1511–1522.

9. Salomon, D., O. Ayalon, R. Patel-King, R.O. Hynes, and B. Geiger. 1992. Extrajunctional distribution of N-cadherin in cultured human endothelial cells. *J. Cell Sci.* 102:1–11.

10. Dejana, E., M. Corada, and M.G. Lampugnani. 1995. Endothelial cell-to-cell junctions. *FASEB J.* 9: 910–918.

11. Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenic regulators. *Science (Wash.DC).* 251:1451–1455.

12. Geiger, B., and O. Ayalon. 1992. Cadherins. *Ann. Rev. Cell Biol.* 8:307–332.

13. Kemler, R. 1992. Classical cadherins. *Semin. Cell Biol.* 3:149–155.

14. Grunwald, G.B. 1993. The structural and functional analysis of cadherin calcium-dependent cell adhesion molecules. *Curr. Opin.Cell Biol.* 5: 797–805.

15. Ranscht, B. 1993. Cadherins and catenins: interactions and functions in embryonic development. *Curr. Opin. Cell Biol.* 6:740–756.

16. Breviario, F., L. Caveda, M. Corada, I. Martin-Padura, P. Navarro, J. Golay, M. Introna, D. Gulino, M.G. Lampugnani, and E. Dejana. 1995. Functional properties of human VE-cadherin (7B4/cadherin-5) an endothelial specific cadherin. *Arterioscler. Thromb.* 15:1229–1239.

17. Lampugnani, M.G., M. Corada, L. Caveda, F. Breviario, and E. Dejana. 1995. The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, β-catenin, and α-catenin with vascular endothelial cadherin (VE-cadherin). *J.Cell Biol.* 129:203–217.

18. Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* 9:317–321.

19. Tsukita, S., S. Tsukita, A. Nagafuchi, and S. Yonemura. 1992. Molecular linkage between cadherins and actin filaments in cell–cell adherens junctions. *Curr. Opin. Cell Biol.* 4:834–839.

20. Hulsken, J., J. Behrens, and W. Birchmeier. 1994. Tumor-suppressor gene products in cell contacts: the cadherin-APC-armadillo connection. *Curr. Opin. Cell Biol.* 6:711–716.

21. Takeichi, M. 1993. Cadherins in cancer: implications for invasion and metastasis. *Curr.Opin. Cell Biol.* 5:806–811.

22. Frixen, U.H., J. Behrens, M. Sach, G. Eberle, B. Voss, A. Warda, D. Lochner, and W. Birchmeier. 1991. E-cadherin-mediated cell–cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 113:173–185.

23. Vleminckx, K., L. Vakaet, M.M. Mareel, W. Fiers, and F. Van Roy. 1991. Genetic manipulation of E-cadherin expression by epithelial tumor reveals an invasion suppressor role. *Cell*. 66:107–119.

24. Martin-Padura, I., C. de Castellarnau, S. Uccini, E. Pilozzi, P.G. Natali, M.R. Nicotra, F. Ughi, C. Azzolini, E. Dejana, and L. Ruco. 1995. Expression of VE (Vascular Endothelial)-cadherin and other endothelial-specific markers in haemangiomas. *J. Pathol.* 175:51–57.

25. Navarro, P., L. Caveda, F. Breviario, I. Mandoteanu, M.G. Lampugnani and E. Dejana. 1995. Catenin-dependent and independent functions of vascular endothelial cadherin. *J. Biol. Chem.* 270:30965–30972.

26. de Lisser, H.M., H.C. Yan, P.J. Newman, W.A. Muller, C.A. Buck, and S.M. Albelda. 1993. Platelet/endothelial cell adhesion molecule-1 (CD31)-mediated cellular aggregation involves cell surface glycosaminoglycans. *J. Biol. Chem.* 268:16037–16046.

27. Balzac, F., A.M. Belkin, V.E. Koteliansky, Y.V. Bolanov, F. Altruda, L. Silengo, and G. Tarone. 1993. Expression and functional analysis of a cytoplasmic variant of the β1 integrin subunit. *J. Cell Biol.* 121:171–178.

28. Gulino, D., C. Boudignon, L. Zhang, E. Concord, M.J. Rabiet, and G. Marguerie. 1992. Ca^{2+}-binding properties of the platelet glycoprotein IIb ligand-interacting domain. *J. Biol. Chem.* 267:1001–1007.

29. Golay, J., A. Capucci, M. Arsura, M. Castellano, V. Rizzo, M. Introna. 1991. Expression of c-myb and B-myb, but not A-myb, correlates with proliferation in human hematopoietic cells. *Blood.* 77:149–153.

30. Zanetti, A., G. Conforti, S. Hess, I. Martin-Padura, E. Ghibaudi, K.T. Preissner, and E. Dejana. 1994. Clustering of vitronectin and RGD peptides on microspheres leads to engagement of integrins on the luminal aspect of endothelial cell membrane. *Blood.* 84:1116–1123.

31. Del Sal, G., M.E. Ruaro, L. Philipson, and C. Schneider. 1992. The growth arrest-specific gene, gas 1, is involved in growth suppression. *Cell.* 70: 595–607.

32. Vestal, D.J., and B. Ranscht. 1992. Glycosyl Phosphatidylinositol-anchored T-cadherin mediates calcium-dependent, homophilic cell adhesion. *J.Cell Biol.* 119:451–456.

33. Breier, G., F. Breviario, L. Caveda, R. Berthier, H. Schnurch, U. Gotsch, D. Vestweber, W. Risau, and E. Dejana. 1996. Molecular cloning and expression of murine vascular endothelial-cadherin in early-stage development of cardiovascular system. *Blood.* 87: 630–641.

34. Muller, W.A., C.M. Ratti, S.L. McDonnell, and Z.A. Cohn. 1989. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. *J. Exp. Med.* 170:399–414.

35. Lampugnani, M.G., M. Resnati, E. Dejana, and P.C. Marchisio. 1991. The role of integrins in the maintenance of endothelial monolayer integrity. *J. Cell Biol.* 112:479–490.

36. Arsura, M., M. Introna, F. Passerini, A. Mantovani, and J. Golay. 1992. B-myb antisense oligonucleotides inhibit proliferation of human hematopoietic

cell lines. *Blood.* 79:2708–2716.

37. Ozawa, M., M. Ringwald, and R. Kemler. 1990. Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 87:4246–4250.

38. Nagafuchi, A., and M. Takeichi. 1990. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO J.* 7:3679–3684.

39. Dejana, E., M.G. Lampugnani, M. Giorgi, M. Gaboli and P.C. Marchisio. 1990. Fibrinogen induces endothelial cell adhesion and spreading via the release of endogenous matrix proteins and the recruitment of more than one integrin receptor. *Blood.* 75:1509–1517.

40. Rubinfeld, B., B. Souza, I. Albert, O. Muller, S.H. Chamberlain, F.R. Masiark, S. Munemitsu, and P. Polakis. 1993. Association of the APC gene product with β catenin. *Science (Wash.DC.).* 262:1731–1734.

41. Su, L.K., B. Vogelstein, and K.W. Kinzel. 1993. Association of the APC tumor suppressor gene with catenins. *Science (Wash. DC).* 262:1734–1737.

42. Heasman, J., A. Crawford, K. Goldstone, P. Garner-Hamrick, B. Gumbiner, P. McCrea, C. Kintner, C.Y. Noro, and C. Wylie. 1994. Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell.* 79:791–803.

43. Hinck, L., I.S. Nathke, J. Papkoff, and W.J. Nelson. 1994. Beta-catenin: a common target for the regulation of cell adhesion by Wnt-1 and Src signaling pathways. *Trends Biochem. Soc.* 19:538–542.

44. Watanabe, M., A. Nagafuchi, S. Tsukita, and M. Takeichi. 1994. Induction of polarized cell–cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma cell line. *J. Cell Biol.* 127:247–256.

45. Klymkowsky, M.W., and B. Parr. 1995.The body language of the cells: the intimate connection between cell adhesion and behaviour. *Cell.* 83:5–8.

46. Fawcett, J., C. Buckley, C.L. Holness, I.N. Bird, J.H. Spragg, J. Saunders, A. Harris, and D.L. Simmons. 1995. Mapping the homotypic binding sites in CD31 and the role of CD31 adhesion in the formation of interendothelial cell contacts. *J. Cell Biol.* 128:1229–1241.