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Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells

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SUMMARY

Interendothelial junctions play an important role in the regulation of endothelial functions, such as vasculogenesis, angiogenesis, and vascular permeability. In this paper we show that vascular endothelial growth factor (VEGF), a potent inducer of new blood vessels and vascular permeability in vivo, stimulated the migration of endothelial cells after artificial monolayer wounding and induced an increase in paracellular permeability of human umbilical vein endothelial cells (HUVECs). Furthermore, VEGF increased phosphotyrosine labeling at cell-cell contacts. Biochemical analyses revealed a strong induction of VEGF-receptor-2 (flk-1/KDR) tyrosine-autophosphorylation by VEGF which was maximal after 5 minutes and was followed by receptor downregulation. 15 minutes to 1 hour after VEGF stimulation the endothelial adherens junction components VE-cadherin, β-catenin, plakoglobin, and p120 were maximally phosphorylated on tyrosine, while α-catenin was not modified. PECAM-1/CD31, another cell-cell junctional adhesive molecule, was tyrosine phosphorylated with similar kinetics in response to VEGF. In contrast, activation of VEGF-receptor-1 (Flt-1) by its specific ligand placenta growth factor (PlGF) had no effect on the tyrosine phosphorylation of cadherins and catenins. Despite the rapid and transient receptor activation and the subsequent tyrosine phosphorylation of adherens junction proteins the cadherin complex remained stable and associated with junctions. Our results demonstrate that the endothelial adherens junction is a downstream target of VEGFR-2 signaling and suggest that tyrosine phosphorylation of its components may be involved in the the loosening of cell-cell contacts in established vessels to modulate transendothelial permeability and to allow sprouting and cell migration during angiogenesis.

Key words: Endothelial cell, Vascular endothelial growth factor, Adherens junction, Cadherin, Catenin

INTRODUCTION

Vascular endothelial growth factor (VEGF) and its receptors represent a key regulatory system of endothelial growth and differentiation in embryonic development as well as under physiological and pathological conditions in the adult. At least five different VEGF isoforms are known in human, which are derived by alternative splicing of a single gene and differ in their secretion and heparin-binding properties (Poltorak et al., 1997). In contrast to most other angiogenic growth factors, VEGF is an endothelial-specific mitogen and a potent vascular permeability factor (VPF) secreted by tumor cells (Senger et al., 1983; Keck et al., 1989). In addition, VEGF has been reported to stimulate chemotaxis (Waltenberger et al., 1994) and migration (Abedi and Zachary, 1997) of endothelial cells in a time- and dose-dependent manner. The VEGF-receptors are transmembrane receptor tyrosine kinases characterized by seven immunoglobulin-like domains in the extracellular part and a split kinase domain in the cytoplasmic portion. Two high affinity receptors are known, which are coexpressed on endothelial cells but seem to have different functional properties. VEGF-receptor-1 (VEGFR-1; FLT-1), which is expressed on endothelial cells (De Vries et al., 1992) and monocytes (Clauss et al., 1996), does not only bind VEGF but also the related placenta growth factor (PlGF) (Kendall et al., 1994; Park et al., 1994). Data on the biological activity of VEGFR-1 in endothelial cells are limited (Clauss et al., 1996; Barleon et al., 1997), whereas VEGF-receptor-2 (VEGFR-2; flk-1/KDR) has been shown to mediate the mitogenic and chemotactic effects of VEGF (Waltenberger et al., 1994). Additional studies indicate that the signaling effectors utilized by the VEGF-receptors include phosphoinositide 3-kinase, phospholipase C, Src-proteins and MAP kinase (Waltenberger et al., 1994; Cunningham et al., 1995; D'Angelo et al., 1995; Guo et al., 1995; Seetharam et al., 1995), but the endothelial specific responses mediated by VEGFR-1 and VEGFR-2 as well as the downstream signal transduction pathways are not yet fully understood (for reviews see Breier and Risau, 1996; Risau, 1997).

VEGF and its receptors are expressed during embryonic and

tumor angiogenesis (Plate et al., 1992; Breier et al., 1995) as well as in fenestrated vascular beds in the adult (Breier et al., 1992). New blood vessel formation by angiogenesis involves the degradation of extracellular matrix combined with sprouting and migration of endothelial cells from preexisting capillaries. One of the first events that probably occurs during this process is the weakening of stable cell-cell contacts between endothelial cells in the parent vessel and the transition of a quiescent stationary to a dynamic migratory endothelial cell (Ausprunk and Folkman, 1977). The regulation of motility and adhesion of endothelial cells to the underlying extracellular matrix and to each other is therefore an important aspect of angiogenesis. Furthermore, areas of replicating endothelial cells exhibit increased vascular permeability (Caplan and Schwartz, 1973).

The mechanisms responsible for permeability regulation are not entirely clear at present. Recently it has been shown, that VEGF induces fenestrations (Roberts and Palade, 1995, 1997; Esser et al., 1998), which are involved in the modulation of vascular permeability (Levick and Smaje, 1987). Alternatively, VEGF might act on cell-cell junctions, which restrict paracellular flow between endothelial cells. Cell-cell adhesion involves a variety of molecules, including the cadherin-catenin complex and the immunoglobulin superfamily member platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31). The cadherins are single chain transmembrane polypeptides which mediate homophilic, calcium-dependent adhesion and are specifically associated with the adherens junction region. In this membrane domain they form multiprotein complexes with the cytoplasmic catenin proteins. Whereas the armadillo proteins β-catenin, plakoglobin and p120 can directly bind to cadherins, α-catenin associates with the complex via β-catenin or plakoglobin and thereby links it to the actin cytoskeleton (for review see Barth et al., 1997). Vascular endothelial cadherin (VE-cadherin) is selectively expressed in endothelial cells in culture (Lampugnani et al., 1992, 1995) and in situ (Breviario et al., 1995; Breier et al., 1996; for review see Lampugnani and Dejana, 1997). It has previously been demonstrated that VE-cadherin is important for the regulation of endothelial cell permeability, migration and assembly of new blood vessels (Breviario et al., 1995; Navarro et al., 1995; Vittet et al., 1996). This is supported by our recent data showing that the adherens junction components VE-cadherin, p120 and β-catenin were tyrosine phosphorylated in loosely confluent and migrating, but not in tightly confluent endothelial cells (Lampugnani et al., 1997). Increased tyrosine phosphorylation at the sites of cell-cell and cell-matrix contacts has been correlated with a number of biological processes such as cell migration, cell motility and metastatic spread of tumor cells (Volberg et al., 1991, 1992; Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Shibamoto et al., 1994; Kinch et al., 1995; Takeda et al., 1995). Since growth factor receptor tyrosine kinase activation has been shown to be involved in the modulation of cell adhesiveness (for review see Barth et al., 1997; Daniel and Reynolds, 1997), we analysed if a growth factor which affects vascular permeability may reproduce the changes in adherens junctions that mark the functional state of endothelial cells. We therefore investigated the effects of VEGF stimulation on the biochemical and functional properties of adhesion molecules in primary endothelial cells. A recent report (Abedi and Zachary, 1997) provided evidence that VEGF stimulated the tyrosine phosphorylation and recruitment of p125FAK and paxillin, which are involved in the regulation of cell-matrix adhesion. In this paper we show that VEGF stimulation may also affect cell-cell adhesion by a rapid and transient tyrosine phosphorylation of VE-cadherin, β-catenin, plakoglobin, p120 and PECAM-1, suggesting that this is an important early step in the modulation of intercellular contacts during angiogenesis and the regulation of vascular permeability.

MATERIALS AND METHODS

Materials

Laboratory reagents were purchased from Sigma (Deisenhofen, Germany) unless otherwise stated. A rabbit polyclonal serum against mouse VEGFR-2, which also recognizes human VEGFR-2, was a kind gift from Dr Harald App (SUGEN Inc., Redwood City, CA). A polyclonal antibody recognizing human VEGFR-1 was purchased from Santa Cruz (Heidelberg, Germany). Mouse monoclonal antibodies against VE-cadherin clone TEA1.31 (see Lampugnani et al., 1995), PECAM-1 (clone 5F4, M. G. Lampugnani, unpublished results, and clone P-1 from BioGenex, San Ramon, CA), p120, β-catenin, phosphotyrosine and p125FAK (Transduction Labs, Exeter, UK) were used. Rabbit antibodies against α-catenin, β-catenin and plakoglobin were kindly provided by Dr Rolf Kemler (Freiburg, Germany). For immunolabeling, rhodamine- or FITC-conjugated antibodies against rabbit or mouse immunoglobulins (Dianova, Hamburg, Germany and DAKO, Glostrup, Denmark) and FITC-labeled phalloidin (Sigma) were used. Peroxidase-conjugated secondary antibodies (reactive either with rabbit or mouse immunoglobulins) were obtained from Pierce (Rockford, IL) or Amersham (Braunschweig, Germany), respectively.

Cell culture

Human endothelial cells from umbilical veins (HUVECs) were prepared by the method of Jaffe et al. (1973) as modified by Jarrell et al. (1984) and cultured as described in detail elsewhere (Lampugnani et al., 1992, 1995). The cells were used from passages 2-5. Human umbilical cords were kindly donated from the hospitals in the 'Wetterau', Germany.

For comparison of endothelial cells at different cell densities, HUVEC cells were seeded and cultured as described previously (Lampugnani et al., 1995, 1997). Briefly, recently confluent cells had reached confluence no longer than 18 hours before the experiment, contained many cells undergoing mitosis, and were maximally spread to establish continuous contacts. Long-confluent cultures reached confluency 48-72 hours before the experiment and showed only a few remaining mitotic cells.

Immunoprecipitation

HUVEC cells were grown to confluence, washed with serum-free Medium 199 and starved for 6 hours with endothelial cell basal medium (PromoCell, Heidelberg, Germany) supplemented with 1.5% FCS (PAN, Nuernberg, Germany), 10 units/ml heparin, antibiotics and 2 mM glutamine. The cells were stimulated at 37°C with recombinant VEGF165, VEGF164 or PlGF152 (kindly provided by Dr Herbert Weich, Braunschweig, Germany or purchased from R & D, Minneapolis, MN) and pretreated for the last hour before lysis with 0.1-1 mM vanadate. The cells were washed twice in PBS and then solubilized on ice for 20 minutes with lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5 [TBS], 1% Triton X-100) supplemented with a cocktail of phosphatase and proteinase inhibitors (1 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.36 mM phenantrolin) with occasional gentle agitation. For studies on VE-cadherin, PBS additionally contained Ca^{2+} and Mg^{2+},

and the lysis buffer was supplemented with 2 mM $CaCl_2$. The cells were scraped from the culture dishes and the lysates centrifuged at 13,000 rpm for 10 minutes at 4°C. Supernatants corresponding to the same number of cells were either subjected to immunoprecipitation or aliquots were removed and boiled for 5 minutes after addition of 4× concentrated sample buffer (Laemmli, 1970). Immunoprecipitations were performed by adding precleared lysates to Protein A- or G-Sepharose beads (Pharmacia, Freiburg, Germany), to which the appropriate antibodies had been preabsorbed. After incubation for 2 hours at 4°C under continuous mixing, the Sepharose-bound immune complexes were washed four times with lysis buffer (containing phosphatase and proteinase inhibitors), once with the same buffer without detergent and then boiled in reducing sample buffer. As controls, immunoprecipitations with antibody-coupled Sepharose without addition of cell lysates were also performed. For general stimulation of tyrosine phosphorylation the cells were pretreated for 20 minutes at 37°C with the potent tyrosine phosphatase inhibitor pervanadate, prepared by mixing equal volumes of 100 mM vanadate and 200 mM hydrogen peroxide. After incubating the solution for 20 minutes at room temperature the mixture was used at a 1:1,000 dilution.

Selective extractions with Triton X-100

After stimulation with VEGF, confluent HUVEC cultures were washed twice with Ca^{2+}- and Mg^{2+}-containing PBS, followed by a 3 minute extraction with 0.5% Triton X-100 (Boehringer Mannheim, Mannheim, Germany) in TBS. The extraction buffer was collected, centrifuged and the supernatant defined as the Triton-soluble fraction. Following this extraction the cells appeared homogeneously adherent to the culture vessel with well preserved nuclei and cytoskeletal fibers as judged by phase contrast microscopy. The Triton-insoluble components were gently washed twice with TBS containing protease and phosphatase inhibitors and then extracted with 0.5% SDS and 1% NP-40 (Boehringer Mannheim) in TBS with inhibitors for 20 minutes on ice. The extract was collected, vigorously pipetted, centrifuged and the supernatant was used as the Triton-insoluble fraction.

Immunoblotting

Total cellular extracts or immunoprecipitates were separated by SDS-PAGE in 7% gels, transferred onto 0.2 μm nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), blocked with 3% BSA in PBS containing 0.1% Tween-20 and then incubated with the appropriate primary antibodies (1 hour at room temperature or overnight at 4°C). Immunoreactive bands were visualized by using peroxidase-conjugated secondary antibodies and the ECL western blot detection system (Amersham).

Immunofluorescence microscopy

The procedure has been previously described in detail (Lampugnani et al., 1992, 1995, 1997). Briefly, cells on fibronectin coated glass coverslips (13 mm diameter) were fixed, permeabilized and then labeled with either phosphotyrosine or VE-cadherin antibodies. This was followed by incubation with rhodamine-conjugated secondary antibodies in the presence of FITC-labeled phalloidin, and mounted using Mowiol 4-88 (Calbiochem, La Jolla, CA). To preserve phosphorylated residues, HUVEC cells were treated for 7 minutes with pervanadate before fixation as described by Lampugnani et al. (1997).

Migration assay (in vitro wounding)

Confluent cultures of HUVEC cells on glass coverslips were treated as previously described (Lampugnani et al., 1997). Briefly, the cell monolayer was wounded with a plastic tip after medium aspiration, washed and incubated in culture medium with or without VEGF (100 ng/ml) for the indicated time.

Permeability assay

HUVEC cells (2.7×10^4) were seeded on fibronectin-coated Transwell filters (0.4 μm pore size, Costar, Cambridge, MA) in 24-well dishes and cultured with 100 μl Medium 199 with 20% newborn calf serum (growth medium) in the upper chamber and 600 μl growth medium in the lower chamber. The cells were grown for three days without medium change until they had reached confluence. For the assay 5 μl of FITC-dextran (M_r 38.9×10^3, 10×10^3, 4×10^3; Sigma, final concentration 1 mg/ml), FITC-inulin (M_r 3×10^3, Sigma, final concentration 2 mg/ml) or of the paracellular tracer molecule neutral Texas Red-dextran (Wong and Gumbiner, 1997) (M_r 40×10^3, Molecular Probes, Leiden, The Netherlands, final concentration 0.5 mg/ml) were added to the upper chamber. This was immediately followed by addition of 5 μl growth medium with or without VEGF165 (final concentration 100 ng/ml). At the indicated time points 50 μl samples were taken from the lower compartment and replaced with the same volume of growth medium to maintain hydrostatic equilibrium. The samples were diluted to 1 ml with PBS and the fluorescent content was measured at 492/520 nm and 587/610 nm absorption/emission wavelengths for FITC- or Texas Red-conjugated dextran, respectively.

Northern analysis

Total cytoplasmic RNA was isolated from HUVEC cultures using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Equal amounts of RNA (10 μg/lane) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde. Blotting and hybridization was performed as described (Ikeda et al., 1995). The following probes were used for hybridization: human Flt-1 cDNA (Plate et al., 1992), human KDR cDNA (Terman et al., 1991) and for standardization chick β-actin cDNA (Kost et al., 1983). Quantitative analysis was performed with the PhosphoImager BAS-2500 (Fujifilm, Raytest, Straubenhardt, Germany).

RESULTS

VEGF stimulates migration and paracellular permeability in HUVECs

VEGF has been previously shown to stimulate chemotaxis and migration of endothelial cells (Waltenberger et al., 1994; Abedi and Zachary, 1997) and to increase vascular permeability (Connolly et al., 1989). In contrast, cell migration and monolayer permeability of cells transfected with the endothelial-specific adhesion molecule VE-cadherin was significantly reduced (Breviario et al., 1995; Navarro et al., 1995; Caveda et al., 1996). We therefore speculated that VEGF might mediate its effects by modulating cell-cell contact proteins. Previous migration assays have mostly used Boyden chamber assays using single cell suspensions. In order to apply a system more similar to the in vivo situation, in which endothelial cells emigrate from an established capillary tube while still maintaining contact to neighboring endothelial cells, we used the in vitro wounding assay. As has been observed before (Waltenberger et al., 1994; Abedi and Zachary, 1997) VEGF stimulated endothelial cell migration, the formation of actin filaments and increased the number of transversally oriented stress fibers (data not shown) in the wounded region. These effects were already visible 6 hours after VEGF addition and became more pronounced after 20 hours of treatment. Overall, these data indicate that VEGF promotes cell migration and sprouting and thereby affects biological functions, which are regulated by VE-cadherin.

We then studied the physiological effect of VEGF on the integrity of intercellular junctions by measuring the

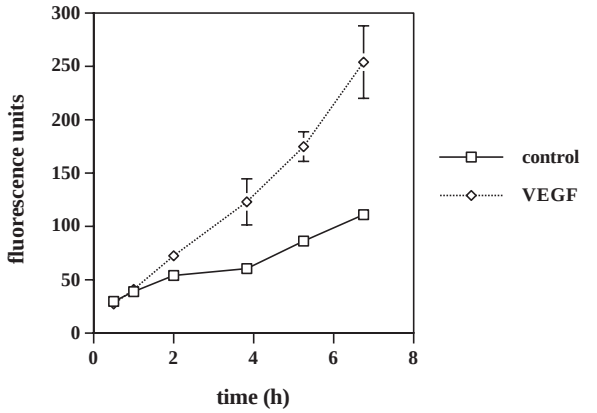

Fig. 1. VEGF stimulates permeability in HUVECs. Confluent layers of HUVEC cells on Transwell filter insets were used. Monolayer permeability after addition of 100 ng/ml VEGF was measured using FITC-dextran (M_r 40×10^3) as described in Materials and Methods. Data are the mean ± s.d. from triplicate samples and are representative of seven independent experiments. The data were compared by Student's *t*-test and showed the following values: 2 hours, $P<0.003$; 3.8 hours, $P<0.01$; 5.25 hours, $P<0.001$; 6.75 hours, $P<0.002$. Control, squares; VEGF, diamonds.

permeability of a confluent endothelial monolayer. Confluent monolayers of human endothelial cells cultivated on transwell filter inserts were treated with VEGF (100 ng/ml) and the permeability of the monolayer for FITC-coupled dextran of various sizes was determined at several time points by measuring the fluorescence intensity of the medium in the lower compartment. In control experiments without VEGF, the level of dextran in the lower compartment increased slowly over time. When the cells were cultured in the presence of VEGF, we observed a significant time-dependent increase in dextran permeability compared to control cells, which was significant after a delay of about 2-4 hours and persisted for at least 7 hours (Fig. 1). Experiments with fluorescent inulin (M_r 3×10^3) or the paracellular tracer molecule neutral Texas Red-coupled dextran (M_r 40×10^3) yielded similar results, although the effects with the latter tracer were less pronounced (data not shown).

Since decreased cell-cell adhesion and increased motility and permeability of epithelial and endothelial cell monolayers has been associated with elevated tyrosine phosphorylation levels of junction components (Shibamoto et al., 1994; Kinch et al., 1995; Staddon et al., 1995) we investigated, if VEGF might transduce these effects in HUVECs by changing the level of phosphotyrosine at intercellular contacts. The phosphotyrosine signal in unstimulated long-confluent monolayers was very diffuse in large overlapping cellular areas and showed only weak staining at focal adhesions (Fig. 2a). VEGF stimulated the phosphotyrosine labeling, which was evident already after 10 minutes (Fig. 2b) and still prominent after several hours of treatment (Fig. 2c,d). In addition to an increase in the immunofluorescence staining of focal adhesions, VEGF induced an upregulation of the phosphotyrosine signal in areas of cell-cell contact which was apparent as a sharp continuous line at intercellular junctions. These data are consistent with our previous observations indicating that phosphotyrosine localization at endothelial junctions might accompany a condition of 'looser', non-compacted cell-cell adhesion (Lampugnani et al., 1997).

Expression of VEGF-receptors in HUVEC

To study whether VEGF induces the alteration of the phosphotyrosine signal at the intercellular junctions by increasing the tyrosine phosphorylation of components of the cell-cell adhesion complex, we investigated the VEGF-induced signaling pathways which might be responsible for these

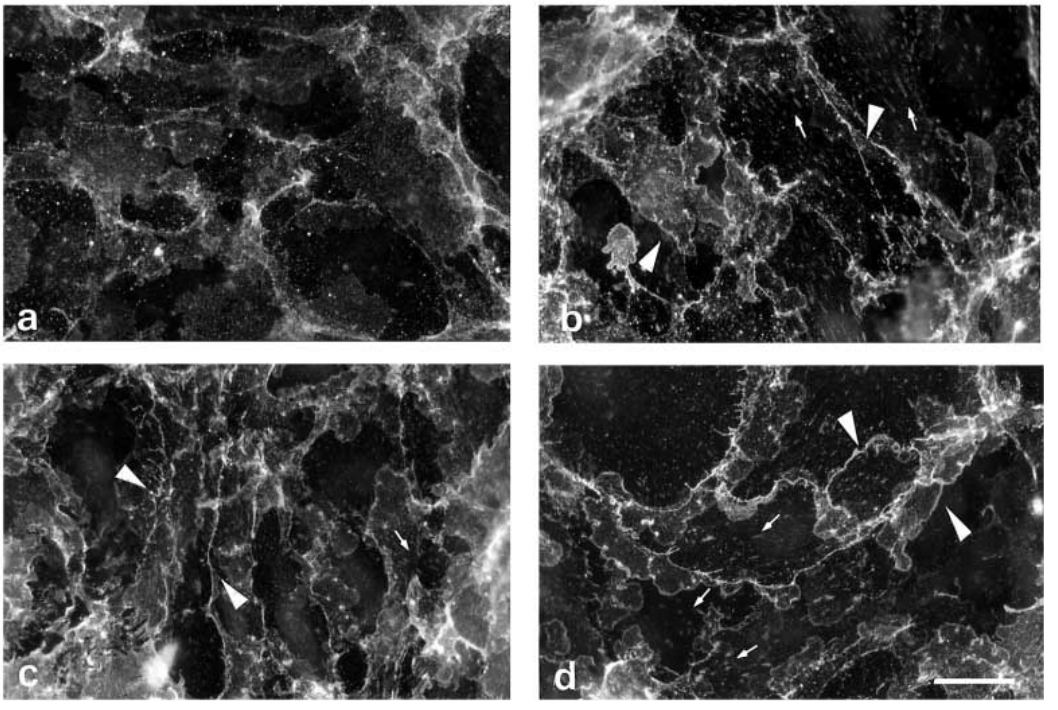

Fig. 2. VEGF induces redistribution of the phosphotyrosine signal at endothelial cell-cell contacts. Confluent cultures of HUVECs were either left untreated (a) or treated with 100 ng/ml VEGF165 for 10 minutes (b), 1 hour (c) or 7 hours (d). The cells were then pretreated with pervanadate for 7 minutes, fixed, permeabilized and incubated with an antibody to phosphotyrosine. Without growth factor addition phosphotyrosine labeling was diffusely visible in large areas of apparent cell overlapping (a). After VEGF treatment (c-d), there was a marked increase in phosphotyrosine signal, which was particularly concentrated in the regions of cell-cell contacts (arrowheads) as well as focal contacts (arrows). Bar, 20 μm.

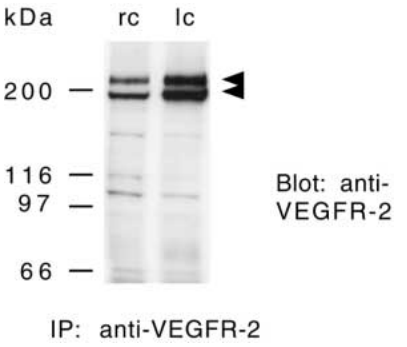

Fig. 3. Expression of VEGFR-2 in HUVECs. Equal numbers of recently confluent (rc) or long-confluent (lc) cultures of HUVEC cells were extracted, immunoprecipitated with anti-VEGFR-2 antibodies and analyzed by immunoblotting with antibody to VEGFR-2. The position of the double band for the receptor is indicated by arrowheads.

effects. Confluent cultures of HUVECs were first examined for the expression of VEGF receptors. Total cell lysates of HUVEC cells at different stages of confluency were analysed by western immunoblotting and northern blotting analysis. The polyclonal antibody recognizing VEGFR-2 detected a double band around 205 kDa in total cell lysates and immunoprecipitates (Figs 3 and 4). Differential glycosylation of the extracellular domain of VEGFR-2 was shown to be responsible for the appearance as a doublet (Takahashi and Shibuya, 1997). The amount of VEGFR-2 protein was increased in long-confluent compared to recently confluent cells (Fig. 3). Northern blotting analysis of total RNA from HUVEC and mouse brain endothelial cells corroborated these results (data not shown). In contrast, immunoblotting with an antibody against VEGFR-1 showed only a very weak expression of VEGFR-1 protein in HUVECs which was also confirmed on the message level by northern blotting analysis (data not shown). In order to distinguish between VEGFR-2

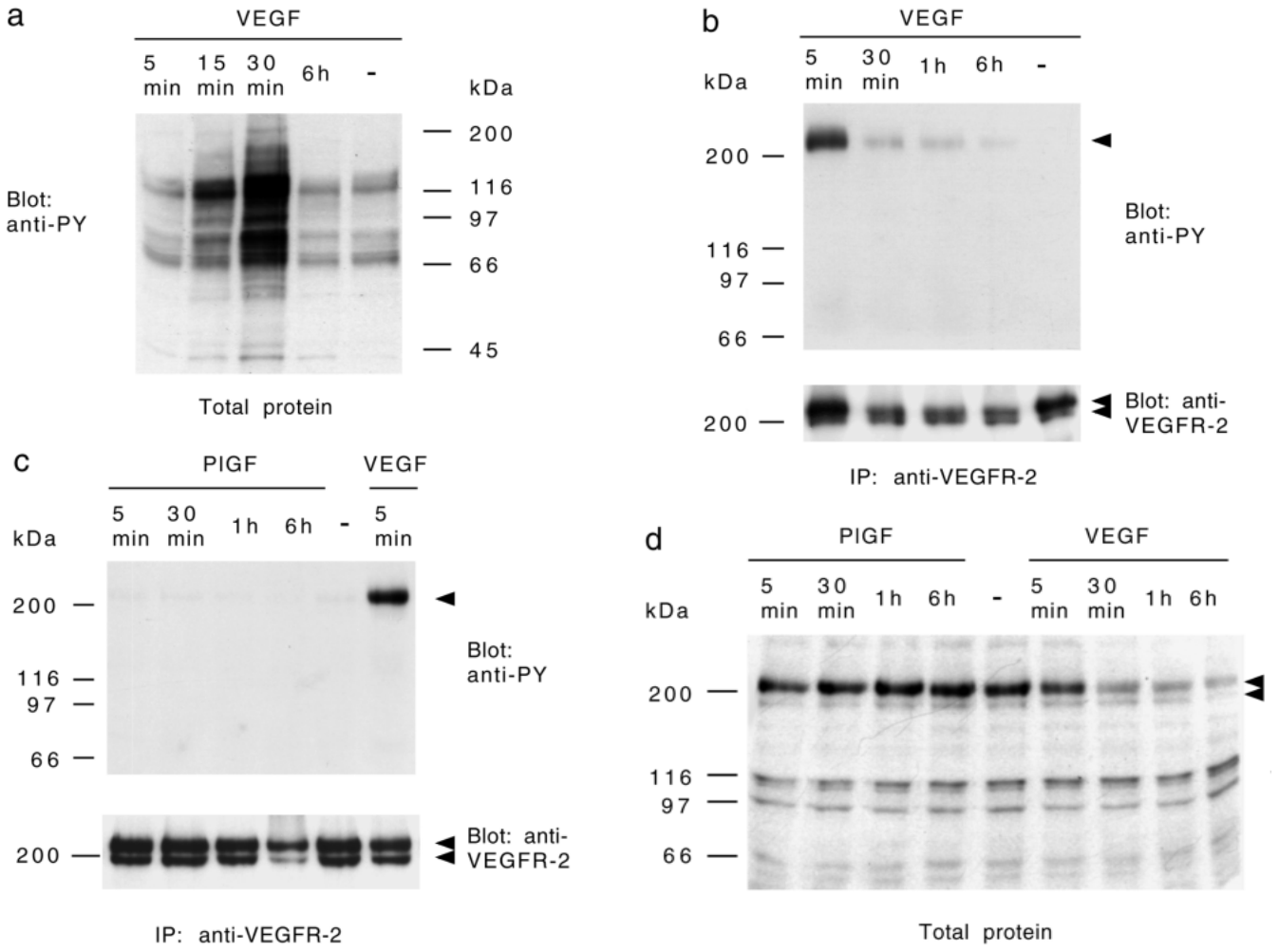

Fig. 4. VEGF stimulates tyrosine phosphorylation of total cellular proteins and VEGFR-2 in HUVECs. Confluent cultures of HUVECs were starved for 6 hours and incubated with 50 ng/ml VEGF165 (a,b), 100 ng/ml VEGF165 (c,d) or 100 ng/ml PlGF152 for different times. Cells were lysed and aliquots of whole cell extracts (a,d) or anti-VEGFR-2 immunoprecipitates (b,c) were analyzed by SDS-PAGE. Immunoblotting analysis was performed with antibodies against phosphotyrosine (a, b and c, top) or VEGFR-2 (d). Blots of immunoprecipitates were reprobed with anti-VEGFR-2 antibody (b and c, bottom). The position of VEGFR-2 is indicated by arrowheads. Note that only the high molecular mass form of VEGFR-2 is tyrosine-phosphorylated in response to VEGF.

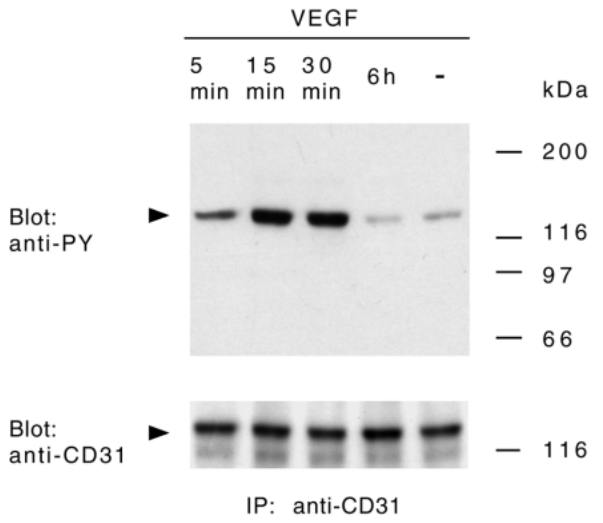

Fig. 5. VEGF stimulates tyrosine phosphorylation of PECAM-1/CD31 in HUVECs. Confluent cultures of HUVECs were starved for 6 hours and incubated with 50 ng/ml VEGF165 for different times. The cells were lysed, anti-PECAM-1/CD31 immunoprecipitates were prepared and immunoblotted with antibody to phosphotyrosine (top). The same blot was then subsequently reprobed with anti-PECAM-1/CD31 antibody (bottom). The position of PECAM-1/CD31 is indicated by an arrowhead.

and VEGFR-1 signal transduction, we used the VEGFR-1 exclusive ligand PlGF152 as a control in the subsequent experiments. In addition, we used long-confluent cells for the further studies, because these cells exhibited established cell-cell contacts (Lampugnani et al., 1995) and expressed high amounts of VEGFR-2.

VEGF stimulation of tyrosine phosphorylation in HUVECs

Concentrations of VEGF known to maximally induce autophosphorylation of VEGF-receptors (Waltenberger et al., 1994) stimulated the tyrosine phosphorylation of multiple proteins in HUVECs with major bands around M_r 205, 160, 120, 100, 80 and 60 (Fig. 4a). Under our culture conditions, VEGF-induced phosphorylation of total cellular proteins was already visible after 5-15 minutes of incubation with a further increase after prolonged stimulation. Maximal phosphorylation was reached after 30 minutes to 1 hour of incubation. After several hours of VEGF-treatment tyrosine phosphorylation of proteins in HUVECs decreased, often to levels below the control values. Most of the tyrosine-phosphorylated proteins were found in the Triton-soluble fraction, whereas the insoluble fraction showed only a weak phosphotyrosine signal (data not shown). In order to examine whether the 205 kDa tyrosine-phosphorylated protein corresponded to one of the VEGF-receptors, we immunoprecipitated VEGFR-2 from VEGF-treated HUVEC cultures. As shown in Fig. 4b, VEGF induced the tyrosine phosphorylation of VEGFR-2. In contrast to the phosphorylation time course of total proteins, tyrosine phosphorylation of VEGFR-2 was maximal after 5 minutes with a rapid decrease after 15 minutes and a further decline to basal levels after several hours. Only the high molecular weight band of VEGFR-2 was found to be phosphorylated, although both forms were recovered by precipitation which is consistent with recent results (Takahashi and Shibuya, 1997). In contrast to stimulation of HUVECs with VEGF, incubation of the cells with PlGF152 neither altered significantly the tyrosine phosphorylation of total proteins (data not shown) nor stimulated tyrosine phosphorylation of VEGFR-2 (Fig. 4c) although it was found to be active in biological assays (see Clauss et al., 1996, and data not shown). As shown in Fig. 4b, the amount of VEGFR-2 decreased after stimulation with VEGF in immunprecipitates as well as whole cell extracts (Fig. 4d) suggesting a rapid downregulation of receptor numbers after stimulation. Already 5 minutes following stimulation, the protein level of VEGFR-2 was reduced compared to unstimulated cells, with a further decline after prolonged incubation. Even 6 hours after VEGF addition, the amount of VEGFR-2 protein was still below control levels. Stimulation with PlGF152 did not show downregulation of VEGFR-2 (Fig. 4d).

VEGF induces tyrosine phosphorylation of junctional proteins

To examine whether VEGF-stimulated cell migration, permeability and elevated phosphotyrosine signals at cell-cell contacts involved modification of cell adhesion proteins, immunoprecipitates of various cell adhesion molecules were immunoblotted with anti-phosphotyrosine antibodies. We focused on two of the main transmembrane cell-cell adhesion systems in endothelial cells; the PECAM-1 molecule and the cadherin adhesion complex. VEGF induced the tyrosine phosphorylation of PECAM-1 in HUVEC cells, with a maximum level of phosphorylation after 15 minutes to 1 hour (Fig. 5). This time course is similar to the phosphorylation kinetics of total cellular proteins, but is delayed with respect to the phosphorylation of VEGFR-2. We next investigated, whether components of the cadherin-catenin adhesion complex were also tyrosine phosphorylated in HUVEC in response to VEGF. As shown in Fig. 6, VEGF stimulated the tyrosine phosphorylation of VE-cadherin as well as of two associated proteins, which were identified as β-catenin and plakoglobin. Although α-catenin was also coprecipitated with VE-cadherin, it was not found to be tyrosine phosphorylated at any time following VEGF treatment (Fig. 6b) nor after pervanadate treatment (data not shown). The effect of VEGF on VE-cadherin phosphorylation was already visible after 5 minutes, but the maximum increase was reached after 15 minutes to 1 hour of incubation time. After 6 hours of stimulation, tyrosine phosphorylation of cadherin and associated catenins had declined. We also examined the effect of VEGF on another VE-cadherin associated molecule, the armadillo protein p120. Immunoprecipitation with an antibody recognizing the two major p120 isoforms in endothelial cells detected two bands around 120 and 100 kDa, with a higher intensity of the isoform at 120 kDa (Fig. 6c). The effect of VEGF on p120 phosphorylation was comparable to its action on VE-cadherin, with a detectable increase in tyrosine phosphorylation as early as 5 minutes after the addition of VEGF, a maximum between 15 minutes to 1 hour and a decrease to control levels after 6 hours of stimulation (Fig. 6c). In most experiments phosphoproteins around 160 kDa of unknown identity were coprecipitated with both p120 and PECAM-1 (data not shown). PlGF152 had no effect on the tyrosine phosphorylation pattern (data not shown).

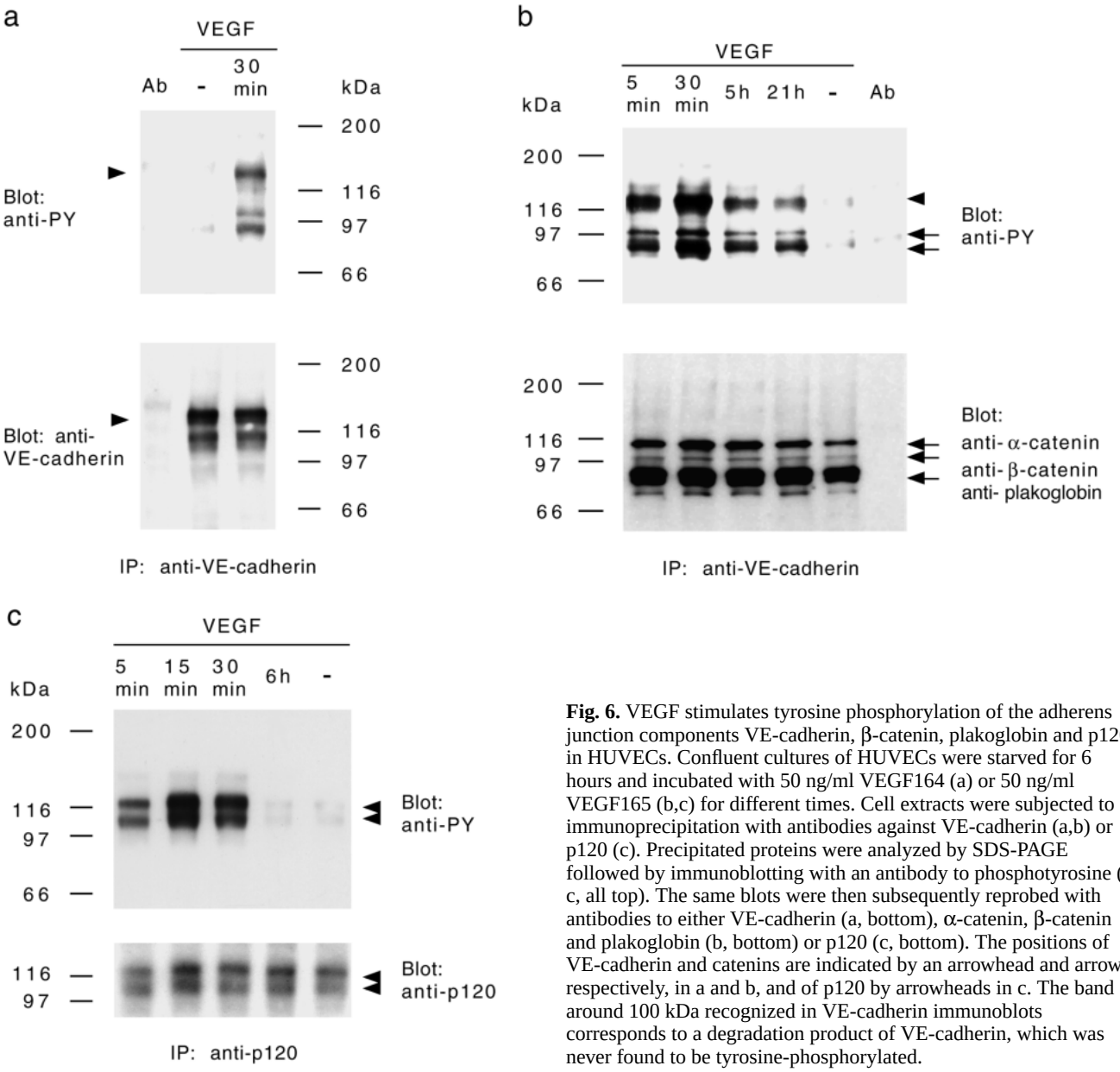

Fig. 6. VEGF stimulates tyrosine phosphorylation of the adherens junction components VE-cadherin, β-catenin, plakoglobin and p120 in HUVECs. Confluent cultures of HUVECs were starved for 6 hours and incubated with 50 ng/ml VEGF164 (a) or 50 ng/ml VEGF165 (b,c) for different times. Cell extracts were subjected to immunoprecipitation with antibodies against VE-cadherin (a,b) or p120 (c). Precipitated proteins were analyzed by SDS-PAGE followed by immunoblotting with an antibody to phosphotyrosine (a-c, all top). The same blots were then subsequently reprobed with antibodies to either VE-cadherin (a, bottom), α-catenin, β-catenin and plakoglobin (b, bottom) or p120 (c, bottom). The positions of VE-cadherin and catenins are indicated by an arrowhead and arrows, respectively, in a and b, and of p120 by arrowheads in c. The band around 100 kDa recognized in VE-cadherin immunoblots corresponds to a degradation product of VE-cadherin, which was never found to be tyrosine-phosphorylated.

Effects of VEGF on junctional proteins

We subsequently examined whether VEGF-induced signal transduction led to changes in the formation or distribution of the cadherin complex. As already shown, tyrosine phosphorylation of VE-cadherin, β-catenin and plakoglobin had no effect on the composition of the cadherin complex, which remained intact even after prolonged VEGF stimulation (Fig. 6a,b). Moreover, the overall level of individual components of the complex as well as the ratio of VE-cadherin to the catenins remained essentially unchanged after VEGF stimulation. This was also true for α-catenin, although it did not become tyrosine phosphorylated by VEGF stimulation. We next investigated, if VEGF stimulation affected the distribution of the cadherin-catenin complex between Triton-soluble and Triton-insoluble compartments in HUVECs. Solubility changes under these conditions are interpreted to indicate altered interactions with the cytoskeleton. A considerable amount of VE-cadherin was extracted under both conditions, but VEGF treatment had no significant effect on the level of VE-cadherin in the detergent soluble or insoluble fractions at all time points tested (Fig. 7). Similar results were obtained for α-catenin, β-catenin, plakoglobin, p120 and PECAM-1 (data not shown). When tyrosine phosphorylation of VE-cadherin and catenins was induced by treatment with pervanadate, the level of VE-cadherin, β-catenin and plakoglobin also remained unaltered (data not shown). The same was true for PECAM-1/CD31 and the unphosphorylated α-catenin protein (data not shown).

To further examine the significance of VEGF action on cadherin-associated junctions, the localization of cadherins and catenins was examined by immunofluorescent labeling. In unstimulated long-confluent HUVEC cells, immunostaining of VE-cadherin, catenins, p120 and PECAM-1 was localized at the cell contact area (Fig. 8a and Ayalon et al., 1994;

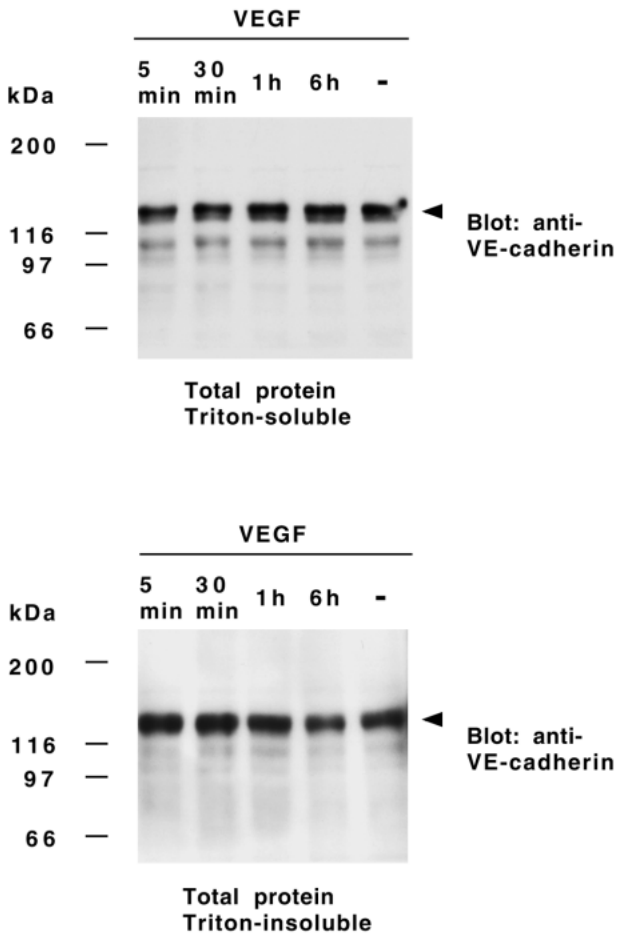

Fig. 7. VEGF does not alter the Triton-solubility of VE-cadherin in HUVECs. Confluent cultures of HUVECs were starved for 6 hours and stimulated with 50 ng/ml VEGF164 for different times. Cells were separated into Triton-soluble (top panel) and -insoluble fractions (bottom panel) as described in Materials and Methods. Aliquots of whole cell lysates were loaded on SDS-PAGE, followed by immunoblotting with a specific antibody to VE-cadherin. The 100 kDa band in the Triton-soluble fractions corresponds to a degradation product of VE-cadherin.

Lampugnani et al., 1995). After VEGF stimulation, the VE-cadherin signal was still visible at intercellular junctions (Fig. 8c,e,g). Although no significant quantitative difference in labeling intensity was discernible, we observed a striking qualitative change in labeling pattern. 10 minutes to 1 hour after VEGF addition, when VEGF-induced cadherin and catenin phosphorylation was maximal, the VE-cadherin staining at cell-cell contacts redistributed in a zigzag pattern, which differed considerably from the straight labeling pattern observed at cellular junctions in control monolayers (Fig. 8a). This redistributed VE-cadherin staining at the cell-cell contacts often appeared to be codistributed with F-actin rich filopodia. In response to VEGF, no gaps were ever visible between the endothelial cells. 7 hours after VEGF incubation (Fig. 8g), this modification was no longer evident and the VE-cadherin staining appeared as a continuous linear labeling at cell adhesions which had almost returned to the pattern seen in control cells.

DISCUSSION

In this study we have shown that VEGF induces a strong increase in tyrosine phosphorylation of the adherens junction components VE-cadherin, β-catenin, plakoglobin, p120 and the cell-cell adhesion molecule PECAM-1 in cultures of confluent HUVEC cells. We have previously demonstrated specific localization of phosphotyrosine signals in junctional areas of recently confluent and migrating cells which were absent in long-confluent resting cells (Lampugnani et al., 1997). Tyrosine phosphorylation of junctional proteins has been associated with loss of integrity of intercellular adhesions (Matsuyoshi et al., 1992; Behrens et al., 1993). As VEGF stimulates migration (Abedi and Zachary, 1997, and this report, data not shown) and permeability, both of which involve destabilization of intercellular junctions, it appears that tyrosine phosphorylation of VE-cadherin and catenins is intimately linked to a functional change of intercellular adhesion.

Under our culture conditions, VEGF stimulation led to activation of VEGFR-2 as indicated by rapid autophosphorylation and the subsequent downregulation of the receptor after longer periods of treatment. Because HUVEC cells express two high affinity receptors for VEGF, VEGFR-1 and VEGFR-2, it was conceivable that also VEGFR-1 contributed to VEGF-mediated activation of cadherin phosphorylation. However, several observations argue against a major role of VEGFR-1 in these processes. First, HUVEC, as well as various other endothelial cells express about ten times more VEGFR-2 than VEGFR-1 (Plouet and Moukadiri, 1990; Vaisman et al., 1990; Bikfalvi et al., 1991; Detmar et al., 1995). Consistent with these data, we observed only very low and variable amounts of VEGFR-1 message and protein in HUVECs by northern and immunoblotting analysis (data not shown). Second, in cells transfected with either receptor alone, only the cells transfected with VEGFR-2 responded to VEGF with changes in cell morphology, actin reorganization, membrane ruffling and chemotaxis (Waltenberger et al., 1994). Third, our experiments using PlGF152, which only binds to VEGFR-1, failed to induce a significant increase in tyrosine phosphorylation of total cellular proteins and of adherens junction components. We therefore conclude, that the observed effects of VEGF in our system are mainly mediated by VEGFR-2, but we cannot exclude the possibility that heterodimers of VEGFR-1 and -2 are involved in these processes.

Our results on the downregulation of VEGFR-2 in HUVECs after VEGF stimulation are probably due to rapid internalization and downregulation of VEGF-occupied receptors in cultured cells (Bikfalvi et al., 1991; Omura et al., 1997). Since PlGF152 had no effect on the expression level of VEGFR-2, ligand-induced receptor activation is a likely prerequisite for this effect. However, in a cerebral slice culture system, VEGF upregulated VEGFR-2 (Kremer et al., 1997). This apparent contradiction might be explained by the very low expression of VEGF-receptors in brain in vivo (Plate et al., 1992; Kremer et al., 1997), whereas most endothelial cells in culture already express high levels of VEGFR-2 under basal conditions, preventing a further upregulation by certain factors.

Tyrosine phosphorylation of adherens junction components after stimulation of endothelial cells with VEGF has not been

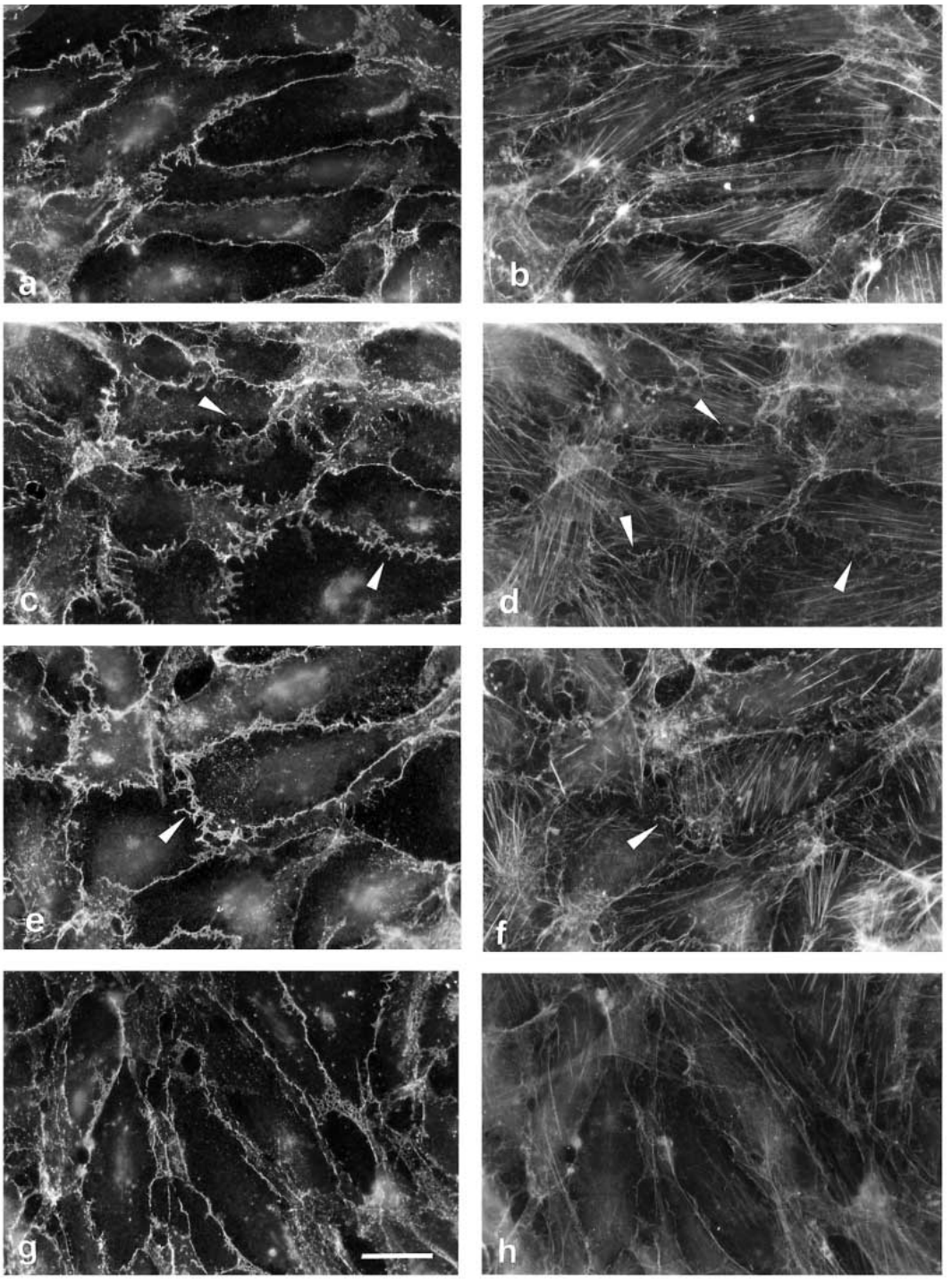

Fig. 8. Effect of VEGF on the junctional localization of VE-cadherin in HUVECs. Confluent cultures of HUVECs were either left untreated (a,b) or treated with 100 ng/ml VEGF165 for 10 minutes (c,d), 1 hour (e,f), or 7 hours (g,h). Cells were fixed, permeabilized and double immunostained for VE-cadherin (a,c,e,g) and F-actin (b,d,f,h). Although VE-cadherin labeling was seen to be localized at cell-cell contacts at all time points tested, the distribution assumed a zigzag pattern codistributing with actin-rich filopodia (arrowheads) 10 minutes (d) to 1 hour (f) after VEGF stimulation. The distribution returned to a more linear pattern similar to the one in control cells 7 hours after VEGF stimulation. Bar, 20 μm.

previously observed. One possible reason might be the high level of tyrosine phosphatases (e.g. PTPγ and HPTPβ) in endothelial cells (Gaits et al., 1995). Since the activity as well as the expression level of several phosphatases has been found to be upregulated by cell density (Pallen and Tong, 1991; Gaits et al., 1995; Gebbink et al., 1995; Fuchs et al., 1996), and since cadherins as well as catenins have been shown to associate with tyrosine phosphatases (Bradykalnay et al., 1995; Balsamo et al., 1996; Fuchs et al., 1996; Kypta et al., 1996), tyrosine phosphorylation of adherens junction components in confluent monolayers of HUVEC might be particularly difficult to detect.

Tyrosine or serine phosphorylation of junctional components has been reported previously for cadherins and the armadillo-catenins after stimulation with growth factors such as EGF, HGF, PDGF and CSF-1 (Downing and Reynolds, 1991; Hoschuetzky et al., 1994; Shibamoto et al., 1994), oncogene-activation (Hamaguchi et al., 1993; Kinch et al., 1995; Takeda et al., 1995), phosphatase inhibition (Volberg et al., 1992; Xu et al., 1997) or TPA-treatment (Nabeshima et al., 1997), and of PECAM-1 after TNF-α treatment, mechanical stimuli or cell spreading and migration (Ferrero et al., 1996; Lu et al., 1996; Osawa et al., 1997). Functionally, increased

tyrosine phosphorylation of β-catenin has been correlated with conditions which perturb cell-cell adhesion (Matsuyoshi et al., 1992; Volberg et al., 1992; Behrens et al., 1993; Shibamoto et al., 1994; Kinch et al., 1995; Takeda et al., 1995), whereas other data indicated that phosphorylation of β-catenin on tyrosine is not required for a shift from the strong to the weak state of adhesion (Takeda et al., 1995). Which functions are influenced by the biochemical modification of the junctional molecules by VEGF? A comparison of the time courses for the VEGF-induced effects in our study suggests a correlation between tyrosine phosphorylation of junctional molecules and the modulation of cell-cell adhesion. In contrast to VEGFR-2 activation, which was maximal after 5 minutes, tyrosine phosphorylation of junctional proteins was visible after 5-15 minutes, and was maximal around 1 hour after VEGF stimulation. Thus, phosphorylation of the cadherin-catenin complex showed similar kinetics as the VEGF-induced tyrosine phosphorylation at cell-cell contacts and the modified junctional distribution of VE-cadherin (Figs 2 and 8). The VEGF induced tyrosine phosphorylation of cell adhesion proteins might also be involved in endothelial migration and paracellular permeability, although a delay is seen in the onset of these biological effects. This might be due to an early triggering of cytoskeletal rearrangements and of the functional modulation of cadherins and catenins, which would lead to changes in adherens junction composition and function at a later stage mediated by as yet unknown pathways. Another function of the modified tyrosine phosphorylation could be the regulation of endothelial proliferation and morphogenesis, since also other growth factors without any primary role in permeability, e.g. EGF, HGF and PDGF, increase the tyrosine phosphorylation of adherens junction components (Downing and Reynolds, 1991; Hoschuetzky et al., 1994; Shibamoto et al., 1994). The weakening of intercellular contacts by tyrosine phosphorylation could therefore be important for the rounding up of cells, cell division and reorganization of the endothelial layer, which all take place during morphogenetic and angiogenic processes. This mechanism is reinforced by our recent observation that tyrosine phosphorylation of endothelial adherens junction components is specifically observed in low density cultures, which are actively proliferating and where the adherens junctions are under reshaping/reorganizing conditions (Lampugnani et al., 1997). Taken together, these results suggest, that VEGF-induced modification of cell contact proteins initiates a transition to a more flexible stage of cell adhesion linked to the destabilization of intercellular contacts.

The mechanisms by which tyrosine phosphorylation might lead to loosening of cell-cell adhesion were investigated here and in previous reports (for review see Daniel and Reynolds, 1997). After VEGF stimulation, the cadherin-catenin complex still remained intact. This has been observed before by several authors (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Reynolds et al., 1994; Shibamoto et al., 1994; Shibata et al., 1994; Papkoff, 1997). In contrast, dissociation of tyrosine phosphorylated β-catenin from the complex has been reported in the case of N-cadherin and E-cadherin (Kinch et al., 1995; Balsamo et al., 1996). In our experiments there was no considerable redistribution of cadherins and catenins between Triton-soluble and Triton-insoluble fractions after stimulation of tyrosine phosphorylation with VEGF. This is consistent with previous data showing that VEGF-induced tyrosine phosphorylation and immunolocalization of p125FAK and paxilllin to focal adhesions (Abedi and Zachary, 1997), and TPA-induced tyrosine phosphorylation of E-cadherin and catenins (Nabeshima et al., 1997) did not change their distribution in these compartments. In contrast, other authors have observed a redistribution to the detergent soluble fraction of β-catenin and p120 after tyrosine phosphorylation (Hoschuetzky et al., 1994; Papkoff, 1997). It is possible that the actual changes in subcellular protein distribution might reflect variations between cell types or differences in the effects of various growth factors and signaling molecules on protein interactions with the cytoskeleton. The finding that VEGF treatment had no significant effect on the junctional localization of VE-cadherin rules out the possibility that increased cell motility and permeability are associated with disappearence of cell adhesion components from the cell-cell contact area. Recently, an interesting mechanism has been proposed (Yap et al., 1997) suggesting that modulation of the adhesion functions of cadherins is independent of their expression level, complex formation with catenins, cytoskeletal activity or signaling, but is regulated by the distribution of cadherin binding sites presented at the cell surface. VEGF-induced modification of adhesion molecules might therefore change the lateral clustering of the extracellular domains of junctional proteins such as VE-cadherin and consequently alter cadherin adhesive strength. It remains to be examined whether the redistribution of VE-cadherin at cell-cell contacts in a zigzag pattern, which has been correlated before with a destabilization of endothelial junctions (Lampugnani et al., 1995; Rabiet et al., 1996) reflects this kind of activity. Alternatively, VEGF may have an effect on the interactions of the cadherin complex with the small GTPases rac and rho, which have been demonstrated to be required for the establishment of cadherin dependent cell-cell contacts (Braga et al., 1997; Takaishi et al., 1997; Tokman et al., 1997). It is also possible that ZO-1, which has been reported to be involved in cadherin-based cell adhesion in non-epithelial cells through its direct binding to α-catenin and actin filaments (Itoh et al., 1997), plays a role in the regulation of VEGF-modulated cell-cell adhesion.

The signaling pathways through which VEGF induces cell migration, chemotaxis and permeability as well as the nature of the kinase(s) responsible for the tyrosine phosphorylation of the junctional proteins remain to be identified. We did not observe any direct association of VEGFR-2 with components of the cadherin-catenin complex, as has been demonstrated for the EGF-receptor and β-catenin (Hoschuetzky et al., 1994). Therefore, VEGF-induced tyrosine phosphorylation of VE-cadherin and catenins might be mediated by recruitment and activation of other tyrosine kinases downstream of the VEGF-receptors. For example, Src-kinases, which are enriched at cell-cell contacts, have been shown to be directly involved in the phosphorylation of cadherins and catenins independently of growth factor receptor activation (Tsukita et al., 1991; Behrens et al., 1993; Reynolds et al., 1994; Papkoff, 1997). Interestingly, Src-kinases have also been identified as components of the VEGF-receptor signaling pathway (Waltenberger et al., 1994).

Taken together, the results presented here indicate that VEGF affects vascular permeability in vitro by inducing

tyrosine phosphorylation of adhesion molecules such as the VE-cadherin complex and PECAM-1/CD31. Since tyrosine phosphorylation of junctional proteins have been implicated in the modulation of cell adhesiveness, we propose that VEGF mediates increased vascular permeability and endothelial migration during angiogenesis at least partially through modulation of VE-cadherin function. Further work is necessary to define the VEGF-dependent signaling pathways and molecules involved in the regulation of cell-cell adhesive functions.

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REFERENCES

Abedi, H. and Zachary, I. (1997). Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J. Biol. Chem*. **272**, 15442-15451.

Ausprunk, D. H. and Folkman, J. (1977). Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* **14**, 53-65.

Ayalon, O., Sabanai, H., Lampugnani, M. G., Dejana, E. and Geiger, B. (1994). Spatial and temporal relationships between cadherins and PECAM-1 in cell-cell junctions of human endothelial cells. *J. Cell Biol*. **126**, 247-258.

Balsamo, J., Leung, T., Ernst, H., Zanin, M. K., Hoffman, S. and Lilien, J. (1996). Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. *J. Cell Biol*. **134**, 801-813.

Barleon, B., Siemeister, G., Martinybaron, G., Weindel, K., Herzog, C. and Marme, D. (1997). Vascular endothelial growth factor up-regulates its receptor fms-like tyrosine kinase 1 (Flt-1) and a soluble variant of Flt-1 in human vascular endothelial cells. *Cancer Res*. **57**, 5421-5425.

Barth, A. I., Nathke, I. S. and Nelson, W. J. (1997). Cadherins, catenins and APC Protein-interplay between cytoskeletal complexes and signaling pathways. *Curr. Opin. Cell Biol*. **9**, 683-690.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M. and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol*. **120**, 757-766.

Bikfalvi, A., Sauzeau, C., Moukadiri, H., Maclouf, J., Busso, N., Bryckaert, M., Plouet, J. and Tobelem, G. (1991). Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: binding, internalization, degradation, and biological effects. *J. Cell Physiol*. **149**, 50-59.

Brady-Kalnay, S. M., Rimm, D. L. and Tonks, N. K. (1995). Receptor protein-tyrosine-phosphatase ptp-mu associates with cadherins and catenins in-vivo. *J. Cell Biol.* **130**, 977-986.

Braga, V. M., Machesky, L. M., Hall, A. and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol*. **137**, 1421-1431.

Breier, G., Albrecht, U., Sterrer, S. and Risau, W. (1992). Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* **114**, 521-532.

Breier, G., Clauss, M. and Risau, W. (1995). Coordinate expression of vascular endothelial growth factor receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. *Dev. Dynam*. **204**, 228-239.

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

Breier, G. and Risau, W. (1996). The role of vascular endothelial growth factor in blood vessel formation. *Trends Cell Biol.* **6**, 454-456.

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

Caplan, B. A. and Schwartz, C. J. (1973). Increased endothelial cell turnover in areas of in vivo Evans Blue uptake in the pig aorta. *Atherosclerosis* **17**, 401-417.

Caveda, L., Martin-Padura, I., Navarro, P., Breviario, F., Corada, M., Gulino, D., Lampugnani, M. G. and Dejana, E. (1996). Inhibition of cultured cell growth by vascular endothelial cadherin (cadherin-5/VE-cadherin). *J. Clin. Invest*. **98**, 886-893.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. **162**, 156-159.

Clauss, M., Weich, H., Breier, G., Knies, U., Rockl, W., Waltenberger, J. and Risau, W. (1996). The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J. Biol. Chem*. **271**, 17629-17634.

Connolly, D. T., Olander, J. V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B. L., Leimgruber, R. and Feder, J. (1989). Human vascular permeability factor. Isolation from U937 cells. *J. Biol. Chem*. **264**, 20017-20024.

Cunningham, S. A., Waxham, M. N., Arrate, P. M. and Brock, T. A. (1995). Interaction of the Flt-1 tyrosine kinase receptor with the P85 subunit of phosphatidylinositol 3-kinase-mapping of a novel site involved in binding. *J. Biol. Chem.* **270**, 20254-20257.

D'Angelo, G., Struman, I., Martial, J. and Weiner, R. I. (1995). Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. *Proc. Nat. Acad. Sci. USA* **92**, 6374-6378.

Daniel, J. M. and Reynolds, A. B. (1997). Tyrosine phosphorylation and cadherin/catenin function [Review]. *BioEssays* **19**, 883-891.

De Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N. and Williams, L. T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* **255**, 989-991.

Detmar, M., Yeo, K. T., Nagy, J. A., Van de Water, L., Brown, L. F., Berse, B., Elicker, B. M., Ledbetter, S. and Dvorak, H. F. (1995). Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. *J. Invest. Dermatol*. **105**, 44-50.

Downing, J. R. and Reynolds, A. B. (1991). PDGF, CSF-1, and EGF induce tyrosine phosphorylation of p120, a pp60src transformation-associated substrate. *Oncogene* **6**, 607-613.

Esser, S., Wolburg, K., Wolburg, H., Breier, G., Kurzchalia, T. and Risau, W. (1998). Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J. Cell Biol*. **140**, 947-959.

Ferrero, E., Villa, A., Ferrero, M. E., Toninelli, E., Bender, J. R., Pardi, R. and Zocchi, M. R. (1996). Tumor necrosis factor alpha-induced vascular leakage involves PECAM1 phosphorylation. *Cancer Res*. **56**, 3211-3215.

Fuchs, M., Muller, T., Lerch, M. M. and Ullrich, A. (1996). Association of human protein-tyrosine phosphatase kappa with members of the armadillo family. *J. Biol. Chem*. **271**, 16712-16719.

Gaits, F., Li, R. Y., Ragab, A., Ragab-Thomas, J. M. and Chap, H. (1995). Increase in receptor-like protein tyrosine phosphatase activity and expression level on density-dependent growth arrest of endothelial cells. *Biochem. J*. **311**, 97-103.

Gebbink, M. F., Zondag, G. C., Koningstein, G. M., Feiken, E., Wubbolts, R. W. and Moolenaar, W. H. (1995). Cell surface expression of receptor protein tyrosine phosphatase RPTP mu is regulated by cell-cell contact. *J. Cell Biol*. **131**, 251-260.

Guo, D., Jia, Q., Song, H. Y., Warren, R. S. and Donner, D. B. (1995). Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. *J. Biol. Chem*. **270**, 6729-6733.

Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. (1993). P60(v-src) causes tyrosine phosphorylation and

inactivation of the n-cadherin catenin cell-adhesion system. *EMBO J*. **12**, 307-314.

Hoschuetzky, H., Aberle, H. and Kemler, R. (1994). Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J. Cell Biol*. **127**, 1375-1380.

Ikeda, E., Achen, M. G., Breier, G. and Risau, W. (1995). Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J. Biol. Chem*. **270**, 19761-19766.

Itoh, M., Nagafuchi, A., Moroi, S. and Tsukita, S. (1997). Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *J. Cell Biol*. **138**, 181-192.

Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest*. **52**, 2745-2756.

Jarrell, B., Levine, E., Shapiro, S., Williams, S., Carabasi, R. A., Mueller, S. and Thornton, S. (1984). Human adult endothelial cell growth in culture. *J. Vasc. Surgery* **1**, 757-764.

Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J. and Connolly, D. T. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246**, 1309-1312.

Kendall, R. L., Wang, G., DiSalvo, J. and Thomas, K. A. (1994). Specificity of vascular endothelial cell growth factor receptor ligand binding domains. *Biochem. Biophys. Res. Commun.* **201**, 326-330.

Kinch, M. S., Clark, G. J., Der, C. J. and Burridge, K. (1995). Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. *J. Cell Biol*. **130**, 461-471.

Kost, T. A., Theodorakis, N. and Hughes, S. H. (1983). The nucleotide sequence of the chick cytoplasmic beta-actin gene. *Nucl. Acids Res*. **11**, 8287-8301.

Kremer, C., Breier, G., Risau, W. and Plate, K. H. (1997). Up-regulation of flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system. *Cancer Res*. **57**, 3852-3859.

Kypta, R. M., Su, H. and Reichardt, L. F. (1996). Association between a transmembrane protein tyrosine phosphatase and the cadherin-catenin complex. *J. Cell Biol*. **134**, 1519-1529.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

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

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

Lampugnani, M. G., Corada, M., Andriopoulou, P., Esser, S., Risau, W. and Dejana, E. (1997). Cell confluence regulates tyrosine phosphorylation of adherens junction components in endothelial cells. *J. Cell Sci*. **110**, 2065-2077.

Lampugnani, M. G. and Dejana, E. (1997). Interendothelial junctions – structure, signalling and functional roles. *Curr. Opin. Cell Biol*. **9**, 674-682.

Levick, J. R. and Smaje, L. H. (1987). An analysis of the permeability of a fenestra. *Microvasc. Res.* **33**, 233-256.

Lu, T. T., Yan, L. G. and Madri, J. A. (1996). Integrin engagement mediates tyrosine dephosphorylation on platelet-endothelial cell adhesion molecule 1. *Proc. Nat. Acad. Sci. USA* **93**, 11808-11813.

Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1992). Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol*. **118**, 703-714.

Nabeshima, K., Asada, Y., Inoue, T., Kataoka, H., Shimao, Y., Sumiyoshi, A. and Koono, M. (1997). Modulation of E-cadherin expression in TPA-induced cell motility: well-differentiated human adenocarcinoma cells move as coherent sheets associated with phosphorylation of E-cadherin-catenin complex. *Lab. Invest*. **76**, 139-151.

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

Omura, T., Miyazawa, K., Ostman, A. and Heldin, C. H. (1997). Identification of a 190-kDa vascular endothelial growth factor 165 cell surface binding protein on a human glioma cell line. *J. Biol. Chem*. **272**, 23317-23322.

Osawa, M., Masuda, M., Harada, N., Lopes, R. B. and Fujiwara, K. (1997). Tyrosine phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) in mechanically stimulated vascular endothelial cells. *Eur. J. Cell Biol*. **72**, 229-237.

Pallen, C. J. and Tong, P. H. (1991). Elevation of membrane tyrosine phosphatase activity in density-dependent growth-arrested fibroblasts. *Proc. Nat. Acad. Sci. USA* **88**, 6996-7000.

Papkoff, J. (1997). Regulation of complexed and free catenin pools by distinct mechanisms. Differential effects of Wnt-1 and v-Src. *J. Biol. Chem*. **272**, 4536-4543.

Park, J. E., Chen, H. H., Winer, J., Houck, K. A. and Ferrara, N. (1994). Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR *J. Biol. Chem.* **269**, 25646-25654.

Plate, K. H., Breier, G., Weich, H. A. and Risau, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* **359**, 845-848.

Plouet, J. and Moukadiri, H. (1990). Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. *J. Biol. Chem*. **265**, 22071-22074.

Poltorak, Z., Cohen, T., Sivan, R., Kandelis, Y., Spira, G., Vlodavsky, I., Keshet, E. and Neufeld, G. (1997). VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. *J. Biol. Chem*. **272**, 7151-7158.

Rabiet, M. J., Plantier, J. L., Rival, Y., Genoux, Y., Lampugnani, M. G. and Dejana, E. (1996). Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization. *Arterioscler. Thromb. Vasc. Biol*. **16**, 488-496.

Reynolds, A. B., Daniel, J., McCrea, P. D., Wheelock, M. J., Wu, J. and Zhang, Z. (1994). Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol. Cell. Biol*. **14**, 8333-8342.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Roberts, W. G. and Palade, G. E. (1995). Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J. Cell Sci*. **108**, 2369-2379.

Roberts, W. G. and Palade, G. E. (1997). Neovasculature induced by vascular endothelial growth factor is fenestrated. *Cancer Res*. **57**, 765-772.

Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S. and Shibuya, M. (1995). A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene* **10**, 135-147.

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S. and Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219**, 983-985.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. and Ito, F. (1994). Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes. Commun.* **1**, 295-305.

Shibata, T., Gotoh, M., Ochiai, A. and Hirohashi, S. (1994). Association of plakoglobin with APC, a tumor suppressor gene product, and its regulation by tyrosine phosphorylation. *Biochem. Biophys. Res. Commun.* **203**, 519-522.

Staddon, J. M., Herrenknecht, K., Smales, C. and Rubin, L. L. (1995). Evidence that tyrosine phosphorylation may increase tight junction permeability. *J. Cell Sci.* **108**, 609-619.

Takahashi, T. and Shibuya, M. (1997). The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts. *Oncogene* **14**, 2079-2089.

Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H. and Takai, Y. (1997). Regulation of cell-cell adhesion by rac and rho small G proteins in MDCJ cells. *J. Cell Biol*. **139**, 1047-1059.

Takeda, H., Nagafuchi, A., Yonemura, S., Tsukita, S., Behrens, J., Birchmeier, W. and Tsukita, S. (1995). V-src kinase shifts the cadherin-based cell adhesion from the strong to the weak state and beta catenin is not required for the shift. *J. Cell Biol*. **131**, 1839-1847.

Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L. and Shows, T. B. (1991). Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* **6**, 1677-1683.

Tokman, M. G., Porter, R. A. and Williams, C. L. (1997). Regulation of

cadherin-mediated adhesion by the small GTP-binding protein Rho in small cell lung carcinoma cells. *Cancer Res*. **57**, 1785-1793.

Tsukita, S., Oishi, K., Akiyama, T., Yamanashi, Y., Yamamoto, T. and Tsukita, S. (1991). Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. *J. Cell Biol*. **113**, 867-879.

Vaisman, N., Gospodarowicz, D. and Neufeld, G. (1990). Characterization of the receptors for vascular endothelial growth factor. *J. Biol. Chem*. **265**, 19461-19466.

Vittet, D., Prandini, M. H., Berthier, R., Schweitzer, A., Martin-Sisteron, H., Uzan, G. and Dejana, E. (1996). Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* **88**, 3424-3431.

Volberg, T., Geiger, B., Dror, R. and Zick, Y. (1991). Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. *Cell Regul.* **2**, 105-120.

Volberg, T., Zick, Y., Dror, R., Sabanay, I., Gilon, C., Levitzki, A. and Geiger, B. (1992). The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO J*. **11**, 1733-1742.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C. H. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.* **269**, 26988-26995.

Wong, V. and Gumbiner, B. M. (1997). A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J. Cell Biol*. **136**, 399-409.

Xu, Y., Guo, D. F., Davidson, M., Inagami, T. and Carpenter, G. (1997). Interaction of the adaptor protein Shc and the adhesion molecule cadherin. *J. Biol. Chem*. **272**, 13463-13466.

Yap, A. S., Brieher, W. M., Pruschy, M. and Gumbiner, B. M. (1997). Lateral clustering of the adhesive ectodomain: a fundamental determinant of cadherin function. *Curr Biol*. **7**, 308-315.