Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, β-catenin, and the phosphatase DEP-1/CD148

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Confluent endothelial cells respond poorly to the proliferative signals of VEGF. Comparing isogenic endothelial cells differing for vascular endothelial cadherin (VE-cadherin) expression only, we found that the presence of this protein attenuates VEGF-induced VEGF receptor (VEGFR) 2 phosphorylation in tyrosine, p44/p42 MAP kinase phosphorylation, and cell proliferation. VE-cadherin truncated in β-catenin but not p120 binding domain is unable to associate with VEGFR-2 and to induce its inactivation. β-Catenin–null endothelial cells are not contact inhibited by VE-cadherin and are still responsive to VEGF, indicating that this protein is required to restrain growth factor signaling. A dominant-negative mutant of high cell density–enhanced PTP 1 (DEP-1)//CD148 as well as reduction of its expression by RNA interference partially restore VEGFR-2 phosphorylation and MAP kinase activation. Overall the data indicate that VE-cadherin–β-catenin complex participates in contact inhibition of VEGF signaling. Upon stimulation with VEGF, VEGFR-2 associates with the complex and concentrates at cell–cell contacts, where it may be inactivated by junctional phosphatases such as DEP-1. In sparse cells or in VE-cadherin–null cells, this phenomenon cannot occur and the receptor is fully activated by the growth factor.

Introduction

VEGF plays an important role in the formation of new vessels during embryogenesis and in proliferative diseases in the adult (Ferrara and Alitalo, 1999; Carmeliet and Jain, 2000; Dor et al., 2002). This effect is mediated by the capacity of the growth factor to induce endothelial cell proliferation and differentiation in vascular structures.

VEGF acts by binding to two high affinity receptor tyrosine kinases: VEGF receptor (VEGFR)* 1 also called Flt-1, and VEGFR-2, also called Flk-1/KDR. Although the role of VEGFR-1 in VEGF signaling is still debated, VEGFR-2 is effective in transducing signals regulating cell division and inhibition of cell death.

Contact-inhibited cells, including the endothelium, have a reduced response to specific growth factors when they reach confluence (Fagotto and Gumbiner, 1996; Vinals and Pouyssegur, 1999). It is likely that the establishment of intercellular contacts transfers negative intracellular signals, which restrains the capacity of the cells to respond to proliferative signals. Cadherins have been implicated in this phenomenon. These adhesive transmembrane proteins are specifically located at intercellular adherens junctions and,
once the cells get in contact, form zipper-like structures along intercellular contacts.

Endothelial cells express an endothelial-specific cadherin called vascular endothelial cadherin (VE-cadherin) (Lampugnani et al., 1992). This protein, like the other members of the family, is linked through its cytoplasmic tail to p120, β-catenin, and γ-catenin (Gottardi and Gumbiner, 2001). β- and γ-catenins, by binding to α-catenin, mediate the anchorage of VE-cadherin to the actin cytoskeleton. In addition, β-catenin, and in some conditions γ-catenin and p120, when released in the cytosol, can translocate to the nucleus and modulate cell transcription (Gottardi and Gumbiner, 2001).

β-Catenin is considered an oncogene and, in tumor cells, can induce transcription of genes important in cell cycle regulation (Polakis, 2000; Conacci-Sorrell et al., 2002; van de Wetering et al., 2002). Cadherins would therefore signal in an indirect way by linking β-catenin at the membrane and limiting in this way its translocation to the nucleus.

Epithelial cadherin (E-cadherin) was found to induce contact inhibition of cell growth (St. Croix et al., 1998; Mueller et al., 2000). This effect requires binding of β-catenin to the cadherin cytoplasmic tail (Gottardi et al., 2001; Stockinger et al., 2001) and inhibition of its transcriptional activity.

Several studies indicate that adhesive proteins, such as integrins, can physically interact with growth factor receptors and synergistically modulate cell proliferation, migration, and survival (Schwartz and Baron, 1999). Although less studied, cadherins or the cadherin–catenin complex can interact with growth factor receptors and some effectors of their signaling pathways (Hoschuetzky et al., 1994; Pece and Gutfkind, 2000; Vasioukhin et al., 2001). In endothelial cells, VE-cadherin can associate with VEGFR-2 upon activation of the cells with VEGF. This interaction increases phosphatidylinositol 3 (PI3) kinase activation by the growth factor and improves cell survival (Carmeliet et al., 1999).

In this paper, we show that VE-cadherin exerts a more complex role on VEGF signaling through VEGFR-2. We found that VE-cadherin expression and clustering at intercellular junctions blocks the proliferative response of the cells to this growth factor, and inhibition of VEGFR-2 phosphorylation in tyrosine likely contributes to this effect. We propose that VE-cadherin acts, in part, by forming a complex with VEGFR-2 and bringing the receptor close to junctional phosphatases. VE-cadherin may, therefore, modulate VEGFR-2 signaling by increasing cell survival while inducing contact inhibition of cell growth.

Results

VE-cadherin expression and clustering inhibit VEGF-induced endothelial proliferation

Confluent endothelial cells respond poorly to growth factors as compared with sparse cells (D’Amore, 1992; Vinals and Pouyssegur, 1999), suggesting that intercellular junctions may transfer growth inhibitory signals. To investigate whether VE-cadherin could play a role in this phenomenon, we compared syngenic endothelial cell lines differing for expression of VE-cadherin only. VE-cadherin–null and –positive cells (VEC null and VEC positive, respectively) retained the expression of all the endothelial-specific markers tested (Lampugnani et al., 2002). In addition, transfected VE-cadherin reached levels comparable to wild-type endothelium and was correctly organized at intercellular contacts (Lampugnani et al., 2002).

As shown in Fig. 1 A, VEC-positive cells arrested their growth at confluence, whereas VEC-negative cells maintained a sustained growth rate reaching a two to threefold higher density than positive cells. Confluent VEC-positive cells behaved like wild-type endothelium (Caveda et al., 1996; Vinals and Pouyssegur, 1999), showing a markedly lower DNA synthesis upon VEGF stimulation than sparse cells (Fig. 1 B). In contrast, VEC-null cells were highly responsive to VEGF both in sparse and confluent conditions. Thus, VE-cadherin substantially reduces the response of confluent, but not sparse, endothelium to VEGF.

Figure 1. Confluence and VE-cadherin expression inhibit endothelial proliferation induced by VEGF. (A) Growth curve of endothelial cells expressing (VEC positive) or not expressing (VEC null) VE-cadherin. Cells (seeding 30,000/cm2) were cultured in complete culture medium. At the indicated time point, cells were detached and counted. The standard deviation in three independent experiments was between 5 and 10% of the mean values. (B) Confluent (100,000/cm2) and sparse (20,000/cm2) VEC-positive and VEC-negative cells were stimulated with VEGF (80 ng/ml) for 24 h. BrdU (30 μM) was added during the last 4 h. A total of 300 nuclei in random fields for each treatment were scored. The mean values of three independent experiments ± SD are shown.
VE-cadherin and VEGF

kinase activation (Takahashi et al., 1999b, 2001). In Fig. 2 A, we show that the presence of VE-cadherin markedly reduced tyrosine phosphorylation of VEGFR-2 after VEGF activation of the cells. We then tested whether the correct clustering of VE-cadherin at junctions, and not only its expression, could affect receptor activation. As shown in Fig. 2 B, sparse VEC-positive cells were able to respond to VEGF like null cells. Similarly, when freshly isolated human umbilical vein endothelial cells (HUVEC) were tested, VEGFR-2 phosphorylation was higher in sparse than in confluent cells (Fig. 2 C).

Figure 2. VE-cadherin expression and clustering inhibit VEGFR-2 tyrosine phosphorylation. (A) Confluent VEC-null and -positive endothelial cells were stimulated with VEGF (80 ng/ml) for the indicated time intervals. Cell extracts were immunoprecipitated with antibodies to VEGFR-2 (IP αVEGFR-2) and immunoblotted (IB) with antibodies to phosphotyrosine (αphosphoTyr) and VEGFR-2 (αVEGFR-2). A similar experimental procedure was used for B–D. In the representative experiment shown, tyrosine-phosphorylated VEGFR-2 normalized over total VEGFR-2 was 0.5-, five-, and twofold more in VEC-null than in VEC-positive cells at time 0, 5, and 30 min, respectively. In 15 independent experiments, the range of increase at 5 min was from two- to sevenfold. (B) Tyrosine phosphorylation of VEGFR-2 in response to VEGF (80 ng/ml for 5 min) in sparse VEC-null and -positive endothelial cells was comparable. (C) In HUVEC, phosphorylation of VEGFR-2 in response to VEGF (80 ng/ml for 5 min) was lower in confluent than in sparse cultures (range three- to fivefold lower in four experiments). (D) Addition of antibodies to VE-cadherin (anti-VEC, 100 μg/ml) for 1 h increased receptor phosphorylation by VEGF (80 ng/ml, for 5 min). In response to VEGF, the phosphotyrosine content in VEGFR-2 was higher (from three- to fourfold in three experiments) in cells pretreated with VE-cadherin antibody. The antibody to VEGFR-2 recognized two bands at a molecular mass of ~200 kD. Only the higher molecular mass band, representing the mature form of the receptor, was phosphorylated in tyrosine, as also described by Takahashi and Shibuya (1997). In the following figures, we therefore show only the heavier band of the doublet, which represents the phosphorylatable pool of VEGFR-2.

Figure 3. VE-cadherin expression and clustering reduce the extent of p44/42 MAP kinase phosphorylation in response to VEGF. (A) Confluent and sparse VEC-null and VEC-positive endothelial cells were stimulated with VEGF (80 ng/ml for 10 min), and phosphorylation of p44/42 MAP kinase and total MAP kinase were assayed by Western blot with specific antibodies. The columns represent the ratio between phosphorylated and total values as fold increase over the ratio calculated in sparse unstimulated VEC null. VEGF-stimulated phosphorylation of MAP kinases was reduced at confluence only in VEC-positive cells. The mean ± SD of three independent experiments is reported. In a total of 12 experiments, the increase of p44/42 MAP kinase phosphorylation in VEC-null cells ranged from three- to sixfold over VEC-positive cells. (B) In confluent HUVEC, phosphorylation of p44/42 MAP kinase in response to VEGF (80 ng/ml for 10 min) was reduced about threefold in comparison with sparse cells. Column values are as in A.
We have previously reported that the addition of VE-cadherin blocking antibodies to confluent endothelial cells abolished localization and clustering of the protein at intercellular contacts (Corada et al., 2002). This effect was not accompanied by cell retraction or redistribution of other junctional proteins (Corada et al., 2001, 2002). As shown in Fig. 2D, the addition of a blocking VE-cadherin monoclonal antibody to confluent HUVEC induces a three to fourfold increase in VEGFR-2 phosphorylation. Together, these data indicate that not only VE-cadherin expression, but also its correct organization and clustering at junctions, is required for an optimal inhibitory effect on VEGFR-2 phosphorylation.

VEGF-R2 was similarly accessible to its ligand in confluent VEC-null and -positive cells. When a monoclonal antibody to the VEGF binding domain of the receptor (DC101) (Witte et al., 1998) was added in vivo to both cell types, it could recognize a comparable amount of VEGFR-2 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200209019/DC1).

VE-cadherin counteracts p44/42 MAP kinase phosphorylation induced by VEGF

Activation of MAP kinases is an important event in endothelial cell proliferation induced by growth factors (Vinals and Pouyssegur, 1999). As reported in Fig. 3A and Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200209019/DC1), in confluent but not in sparse cells, VE-cadherin expression reduced the peak and duration of MAP kinase phosphorylation induced by VEGF. Consistently, upon VEGF stimulation, sparse HUVEC presented higher MAP kinase activation than confluent cells (Fig. 3B).

Treatment of VEC-null and -positive cells with PD98059 significantly reduced p44/42 MAP kinase activation and DNA synthesis after VEGF (Fig. 4, A and B). In addition, transfection of VEC-positive cells with a constitutively active MAP kinase construct or the empty vector, partially but significantly rescued cell proliferation.

Changing VEGF concentrations, both VEGF-R2 and p44/42 MAP kinase phosphorylation presented a dose-dependent response (Fig. 5). VEC-null cells always presented higher receptor phosphorylation and MAP kinase activation at all concentrations of VEGF used.

VE-cadherin forms a complex with VEGFR-2

In previous work (Carmeliet et al., 1999), we found that VE-cadherin can be coimmunoprecipitated with VEGFR-2. We investigated whether this interaction is required for inhibition of VEGFR-2 phosphorylation. To this end, we analyzed VEC-null endothelial cells transfected with different mutants of VE-cadherin cytoplasmic tail. As described previously in detail (Lampugnani et al., 2002), mutant proteins are expressed at levels comparable to the wild type and are correctly clustered to cell–cell contacts. As reported in Fig. 6 (A and C), when the region responsible for binding to β-catenin was truncated (from aa 703 to 784, Δβcat), VEGFR-2 could not associate with VE-cadherin. In contrast, when the region responsible for binding to β-catenin was truncated (from aa 621 to 702, Δp120), the receptor was still coimmunoprecipitated with VE-cadherin. Compared with null cells, expression of Δβcat did not change VEGFR-2 phosphorylation, whereas transfection of Δp120 reduced this parameter, although not as effectively as VE-cadherin wild type (Fig. 6A).

The differences in receptor phosphorylation paralleled those in cell proliferation. Fig. 6B shows that Δβcat transfectants responded to VEGF as null cells, whereas Δp120 cells were partially inhibited. Coimmunoprecipitation experiments confirmed that Δβcat and Δp120 VE-cadherin mutants were unable to bind β-catenin or p120, respectively (Lampugnani et al., 2002).

β-Catenin is required for inhibition of VEGFR-2 phosphorylation

To investigate directly the role of β-catenin in VEGFR-2 phosphorylation, we tested β-catenin–null endothelial cells.
These cells were isolated and cultured from animals carrying a null mutation of the β-catenin gene in endothelial cells specifically. Conditional gene inactivation was obtained by crossing mice carrying floxed β-catenin gene (Brault et al., 2001) with transgenics expressing Cre under an endothelial-specific promoter (Tie-2 Cre) (Kisanuki et al., 2001; unpublished data; see Materials and methods for details).

As reported in Fig. 7 (A and B), β-catenin was absent in these mutants whereas VE-cadherin expression was comparable. β-Catenin–null cells retained the endothelial cell markers tested (including VEGFR-2, Tie2, and CD34) and correctly organized junctions (as detected by the localization of VE-cadherin, ZO-1, occludin, PECAM, and JAM) (Fig. 7 A; unpublished data). β-Catenin–null cells presented higher density at confluence and elongated morphology in comparison with the positive cells (Fig. 7 A).

In contrast to positive cells, when β-catenin–null cells were challenged with VEGF, VEGFR-2 could not be coimmunoprecipitated with VE-cadherin (Fig. 7 D) and its phosphorylation was markedly increased (Fig. 7 C). Consistently, endothelial cell proliferation in response to VEGF was increased in β-catenin–null cells (Fig. 7 E). Comparable results were obtained using another β-catenin–null and another control cell line isolated from independent mice.

To test whether Cre expression may contribute to the observed functional behavior of β-catenin–null cells, we infected control cell lines with an adenoviral vector containing Cre cDNA as described previously (Anton and Graham, 1995). Using two adeno Cre infections at 2 pfu/cell concentration at a 9-h interval, we did not observe significant changes in cell growth or response to VEGF (unpublished data). Overall, these data indicate that β-catenin expression and binding to VE-cadherin is required for inhibition of VEGFR-2 phosphorylation and down-regulation of cell proliferation.

**Inhibition of VEGFR-2 phosphorylation is due to phosphatase activity: the role of DEP-1/CD148**

Treatment of VE-cadherin–positive cells with pervanadate (PV; from 100 μM vanadate and 200 μM H₂O₂), a general inhibitor of phosphatases (Volberg et al., 1992), restored the capacity of VEGF to induce VEGFR-2 phosphorylation at an even higher level than VE-cadherin–null cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200209019/DC1). These data link the inhibitory role of VE-cadherin to the activation or localization of phosphotyrosine phosphatase (PTP) activity.

Among different PTPs possibly implicated in this process, we studied the role of high cell density–enhanced PTP1/CD148. This receptor-like PTP is of particular interest because it is up-regulated by cell density (Ostman et al., 1994), is expressed in endothelial cells of different origin, and, most importantly, codistributes with VE-cadherin at interendothelial junctions (Takahashi et al., 1999a).

To investigate the role of DEP-1 in VEGFR-2 phosphorylation, we transfected VE-cadherin–positive cells with either wild-type DEP-1 or a dominant-negative form of this PTP (DEP C/S). These constructs have been previously character-
ized in detail for expression and biological activity (Kume et al., 1996; Trapasso et al., 2000; Baker et al., 2001). In addition, VE-cadherin–positive cells have been targeted with DEP-1–directed short interfering RNA (siRNA) or corresponding scramble oligonucleotides. None of the transfected cell lines presented changes in the morphology, retraction, or junctional distribution of VE-cadherin and β-catenin (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200209019/DC1).

As reported in Fig. 8, expression of VE-cadherin increased DEP-1 protein levels by 80–90% by Western blot analysis. Transfection of VE-cadherin–positive cells with wild-type and mutant DEP-1 constructs resulted in increased levels of the corresponding proteins by Western blot (≈50% in comparison with VEC positive). siRNA directed against DEP-1 reduced DEP-1 production by ≈40% in VE-cadherin–positive cells, whereas the scramble oligonucleotides did not have a significant effect.

We then tested the effect of DEP-1 on VEGFR-2 phosphorylation. As shown in Fig. 9, DEP-1 C/S and siRNA partially restored VEGF-induced receptor phosphorylation in VE-cadherin–positive cells. Wild-type DEP-1 (DEPwt) and the scrambled oligonucleotides slightly, but not significantly, reduced VEGF-2 activation. We obtained similar results when we measured the phosphorylation of Tyr 996 or 951 of the receptor (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200209019/DC1).

Both DEP-1 C/S and siRNA had a partial, but significant, effect in restoring MAP kinase activation and BrdU uptake induced by VEGF in VEC-positive cells (Fig. 10, A and B). Wild-type DEP-1 (DEPwt) and the scrambled RNA did not have any effect.

Finally, we tested whether the effect of DEP-1 inhibition could increase phosphorylation of the receptor also in sparse cells, suggesting a nonspecific, general effect of the phosphatase. As reported in Fig. S6 (available at http://www.jcb.org/cgi/content/full/jcb.200209019/DC1), the effect of DEP-1 C/S was apparent only in confluent cells, and no further increase in the phosphorylation of VEGFR-2 was detected in sparse cultures.

**Discussion**

In this paper, we report that VE-cadherin expression is responsible, to a substantial extent, for the reduced response of
confluent endothelial cells to VEGF-induced proliferation. VE-cadherin–positive or –negative cells respond equally well to the growth factor when they are sparse, but only VE-cadherin–positive cells become insensitive to VEGF at confluence. This indicates that the difference is not mediated by VE-cadherin expression, per se, but requires the establishment of intercellular contacts and cadherin clustering at junctions.

Other papers showed that expression of cadherins could strongly limit cell growth. E-cadherin and N-cadherin in different tumor cell lines induced contact inhibition of cell growth (St. Croix et al., 1998; Levenberg et al., 1999; Gottardi et al., 2001; Stockinger et al., 2001). This effect is mediated, at least in part, by the induction of cell cycle arrest at G1 phase likely due to dephosphorylation of retinoblastoma protein, elevation of the cyclin D1–dependent kinase inhibitor p27kip1, and late reduction of cyclin D1.

The growth inhibitory effects are believed to be mediated by the capacity of cadherins to link β-catenin at the membrane, limiting in this way its nuclear translocation. β-Catenin up-regulates transcription of cyclin D1 and c-myc (Polakis, 2000; Gottardi and Gumbiner, 2001; van de Wetering et al., 2002; Conacci-Sorrell et al., 2002), and inhibition of its activity would limit growth.

However, few observations do not perfectly fit in this picture. The addition of cadherin blocking antibodies restores growth in contact-inhibited cells (Caveda et al., 1996; St. Croix et al., 1998) but does not change the cadherin–β-catenin complex (St. Croix et al., 1998; Corada et al., 1999). Similarly, in sparse or confluent cells, the VE-cadherin–β-catenin complex remains unchanged despite profound differences in growth (Lampugnani et al., 1997).

In this paper, we propose an additional mechanism through which cadherin expression may modulate growth. We found that VE-cadherin expression in confluent endothelial cells is accompanied by a strong reduction of VEGFR-2 phosphorylation. This effect was not due to down-regulation of VEGFR-2 phosphorylation because equal amounts of the protein were found in VE-cadherin–positive and –negative cells or in sparse and confluent cells. The accessibility of the receptor to its ligand, measured as binding of a mAb directed to the active site, was also unchanged.

VE-cadherin clustering at junctions was, however, necessary. The addition of anti–VE-cadherin antibodies, able to disrupt VE-cadherin clusters and induce a diffuse redistribution of the protein on the cell membrane, restores VEGFR-2 phosphorylation.
phosphorylation. In addition, VE-cadherin–positive, sparse cells behave like VE-cadherin–null cells. These data are consistent with previous work by Rahimi and Kazlauskas (1999), who showed that both removing divalent cations or adding VE-cadherin antibodies increased VEGER-2 phosphorylation in endothelial cells. In addition, Takahashi and Suzuki (1996) reported that in human breast epithelial cells, inhibition of E-cadherin clustering restored EGF receptor phosphorylation and cell response to this growth factor.

We published previously that VE-cadherin can form a complex with VE-GER-2 upon activation of the cells with VEGF, and this paralleled VEGER-2 localization at intercellular junctions (Carmeliet et al., 1999; Shay-Salit et al., 2002; Zanetti et al., 2002). We therefore investigated whether the association of VE-cadherin with VEGER-2 influences its phosphorylation. When the cytoplasmic tail of VE-cadherin is truncated in the β-catenin–binding domain, the complex cannot form and receptor phosphorylation is not inhibited. In contrast, when VE-cadherin is truncated in the p120 binding domain, it can still associate with VEGER-2 like the wild type. Receptor phosphorylation in these cells was decreased at 5 min and lasted for a shorter time in comparison with VE-cadherin–null cells. This effect paralleled a reduction in VEGF-induced cell proliferation in Δ-p120 compared with VE-cadherin–null cells. The data discussed above suggested a role of cadherin-associated β-catenin in the inhibition of VEGER-2 activation. This was further confirmed using β-catenin–null cells. These cells presented normal levels of VE-cadherin and all the other endothelial cell markers tested. In addition, VE-cadherin clustering at junctions as well as its association with γ-catenin/plakoglobin were retained. However, in these cells, VEGF association with VE-cadherin and its phosphorylation were not inhibited, and cells could still respond to the proliferative signal of VEGF.

Together, these data indicate that β-catenin is necessary for VEGF-2 dephosphorylation and for down-regulation of cell growth. This observation is somehow in contrast with the general concept that β-catenin expression would favor, and not contrast, cell proliferation. Indeed, there may be cell-specific differences in the response to β-catenin among different cell types and among transformed and nontransformed cells.

**Figure 8.** Expression of tyrosine phosphatase DEP-1 in VEC-positive and -negative cells and effect of transfection of wild-type and DEP-1 C/S constructs and DEP-1 RNAi. In VEC-positive endothelial cells (third lane), DEP-1 was 80–90% higher than in VEC null (fourth lane). In VEC-positive cells, transfection of wild-type (wt, first lane) or point-mutated (C/S, second lane) DEP-1 constructs resulted in increased expression of the protein (50–60% more than in control VEC positive). siRNA directed against DEP-1 (DEP siRNA) resulted in 35–40% inhibition of DEP expression (fifth lane). Scramble oligonucleotides (scr siRNA), used as control (100 nM as DEP siRNA oligonucleotide), were ineffective. Plus and minus indicate the presence or the absence of the protein indicated on the left. The columns report the mean of four experiments ± SD.

**Figure 9.** Down-regulation of DEP-1 increases VEGF-2 phosphorylation. Transfection of the dominant-negative DEP C/S mutant or DEP siRNA induced higher phosphorylation of VEGF-2 upon VEGF (80 ng/ml for 5 min) than the respective controls, VEC positive, DEP wild type (DEP wt), and scramble siRNA (scr siRNA), respectively. Data were obtained by immunoprecipitation (IP) with anti-VEGF-2 antibodies (αVEGF-2) followed by Western blot (IB) with an antiphosphotyrosine antibody (αphosphoTyr). The graph was obtained by quantifying the gel bands and calculating the ratio of bands labeled with antiphosphotyrosine antibodies over total VEGF-2. Each column represents the fold increase over unstimulated cells. Data are means ± SD of four experiments. Plus and minus indicate the presence or the absence of the protein indicated on the left.
formed cells as previously reported by Posthaus et al. (2002). VE-cadherin–null, Δ-βcat, or β-catenin–null mutant cells behave similarly, suggesting that the effect on VEGFR-2 inactivation is related more to localization of β-catenin at the membrane than its nuclear translocation, which cannot occur in β-catenin–null cells. An attractive hypothesis is that the mechanism of action of the VE-cadherin–β-catenin–p120 complex comprises tethering VEGFR-2 to specific phosphatases, inhibiting in this way the degree and duration of its phosphorylation. This is supported by the observation that the general phosphatase inhibitor pervanadate blocks receptor inactivation by VE-cadherin.

Several phosphatases (PTPα, PTP-K, SHP-1, SHP-2, PTP-LAR, and PTP-1B) (Brady-Kalnay et al., 1995; Balsamo et al., 1996; Fuchs et al., 1996; Kypta et al., 1996; Ukropec et al., 2000; Tonks and Neel, 2001) have been found to be directly or indirectly associated with the cadherin–catenin complex. Among these phosphatases, DEP-1/CD148 (Ostman et al., 1994) is of particular interest in endothelial cells. This type I membrane-associated phosphatase is expressed in many endothelial cell lines from different sources, is up-regulated by confluence, and is located at intercellular junctions (Takahashi et al., 1999a). Inactivation of its encoding gene induces a vascular phenotype characterized by enlarged, oversized vessels with abnormally high endothelial cell proliferation (Takahashi et al., 2003). This suggests that the lack of DEP-1 activity leads to uncontrolled endothelial cell growth, and the data reported here may explain these observations. We show that DEP-1 contributes to VE-cadherin–induced inhibition of VEGFR-2 activation. When its activity or expression is inhibited, VEGFR-2 phosphorylation, activation of p44/42 MAP kinase, and cell proliferation are increased.

In previous papers, DEP-1 was found to codistribute with VE-cadherin at endothelial cell junctions; however, chelation of ions and disruption of VE-cadherin clusters did not change DEP-1 distribution (Takahashi et al., 1999a). This suggests that this phosphatase is contiguous but not directly associated with VE-cadherin. We propose that VE-cadherin binding to VEGFR-2 concentrates the receptor at junctions and makes it available to DEP-1 and possibly other junction-associated phosphatases that would limit its activation. The partial rescue of a functional response to VEGF by dominant-negative DEP-1 and DEP-1–directed RNA interference (RNAi) (as well as constitutively activated MAPK kinase) could indicate that other mechanisms besides the effect on VEGFR-2 phosphorylation could be involved in contact inhibition of growth in response to this mitogen. These...
mechanisms could be operated by junctional β-catenin and act in parallel to the direct effect on the receptor.

It has been recently reported that DEP-1 can dephosphorylate the hepatocyte growth factor receptor Met at specific sites. This suggests that this phosphatase has a general role in regulating signaling from growth factor receptors (Palka et al., 2003).

VEGFR-2 triggers different signaling pathways that mediate cell growth and survival. Activation of PLCγ (Takahashi et al., 2001), and in some cases Ras (Meadows et al., 2001), would induce activation of a MAP kinase cascade and cell division. In contrast, PI3 kinase activation and Akt phosphorylation are mostly responsible, with some exception (Dayanir et al., 2001), for promoting cell survival over apoptosis (Gerber et al., 1998a,b).

Inhibition of VEGFR-2 activation by VE-cadherin would repress all its downstream signaling pathways. However, in previous work (Carmeliet et al., 1999), we observed that, instead, expression of VE-cadherin reinforced VEGFR-2 activation of PI3 kinase, accompanied by a marked decrease in endothelial cell sensitivity to apoptotic stimuli. This suggests that the effect of VE-cadherin is more complex and that this protein can direct VEGFR-2 signaling to specific pathways, inhibiting others. A possible explanation is that the phosphatase activity associated with the VE-cadherin–catenin complex may be specific for some VEGFR-2 tyrosines and not others. This would inhibit receptor interaction with some effectors without affecting other pathways. For instance, some authors reported that phosphorylation of tyrosine 1173 is crucial for PLCγ but not for PI3 kinase binding to VEGFR-2 (Takahashi et al., 2001). In contrast, tyrosine 799 constitutes a binding site for the p85 element of PI3 kinase but not for PLCγ (Dayanir et al., 2001). In this paper, we analyzed the behavior of tyrosine 951 and 996 because specific antibodies were available. These two tyrosines contribute to PLCγ and MAP kinase activation. We found that phosphorylation of both tyrosines was restrained by VE-cadherin expression and that this activity was restored by the DEP-1 dominant-negative mutant. A systematic analysis of the phosphorylation of all different tyrosines in VEGFR-2 in the presence or absence of VE-cadherin is underway.

In conclusion, in this paper we report that VE-cadherin expression and clustering may strongly modulate VEGFR-2 signaling. In confluent endothelial cells, VEGFR-2 would preferentially signal through PI3 kinase for survival, whereas in sparse cells, or in cells lacking VE-cadherin, it would mostly promote cell growth through MAP kinase activation. Thus, the same receptor, in the same cells and upon addition of the same agonist, would behave differently if associated or not with VE-cadherin. This introduces the idea that cadherins may contribute to receptor tyrosine kinase signaling by providing a docking platform for binding effectors and inhibitors, which may mediate the diversification of signaling pathways. The absence of VE-cadherin at junctions, as in VE-cadherin–null animals (Carmeliet et al., 1999) or in animals treated with blocking antibodies (Corada et al., 1999), causes profound changes in vascular organization. The lack of modulation of VEGF signaling pathways may play an important role in these effects that lead to early lethality in the embryo and extensive vascular damage in the adult.

Materials and methods

Cell preparation and characterization

Endothelial cells were derived from murine embryonic stem cells with homozygous null mutation of the VE-cadherin gene (VEC null) as described in detail by Balconi et al. (2000). Wild-type and mutant forms of VE-cadherin were introduced in these cells using the retroviral vector PINCO, obtained through the courtesy of P.G. Pelicci (European Institute of Oncology, Milan, Italy) after the authorization of G.P. Nolan (Stanford University, Stanford, CA). Cell types that expressed the following proteins were generated: wild-type-VE-cadherin (VEC positive), Δ-p120 (amino acid deletion 621–702, corresponding to the binding region of p120), and Δ-βcat (amino acid deletion 703–784, corresponding to the binding region of β-catenin). The details of the production and characterization of these cells were described by Lampugnani et al. (2002).

The genes for hamster wild-type MAP kinase (p45 MAPK) and constitutively active MAP kinase kinase (with serine 218 and serine 222 both mutated to aspartic acid; D; Brunet et al., 1994) were also introduced in VEC-positive cells. The constructs, obtained through the courtesy of G. Pages (CNR-S-UMR 6543, Nice, France), were cloned into PINCO vector, which also expressed GFP. Control cells were obtained by transfection with PINCO vector expressing GFP only. After infection, the GFP-positive cells were sorted. Expression of DEP, endogenous and neo-expressed, was assayed by Western blot with a mouse monoclonal antibody to ectodomain (clone D3F) and rabbit polyclonal antibody to cytoplasmic domain, which gave comparable results. Both antibodies recognized the human and mouse form of DEP.

Inhibition for hamster wild-type MAP kinase (p45 MAPK) and constitutively active MAP kinase kinase (with serine 218 and serine 222 both mutated to aspartic acid; D) were isolated from 9.5-d postcoitum littermate embryos, as described in detail by Balconi et al. (2000). Expression of β-catenin protein was tested with the anti-β-catenin antibody by Transduction Laboratories (BD Biosciences), expression of VE-cadherin was tested with BV 13 (Corada et al., 1999), and expression of VEGFR-2 was tested with sc504 by Santa Cruz Biotechnology, Inc. These cells have also been analyzed for the expression of β-catenin mRNA by PCR, expression of other endothelial markers (PECAM, Tie-2, and endoglin), as well as expression and distribution of junctional molecules (α-catenin, plakoglobin, ZO-1, and JAM). For all the endothelial cells of murine origin, culture medium was DME (GIBCO BRL) with 10% fetal calf serum (HyClone), heparin (100 µg/ml), from porcine intestinal mucosa; Sigma-Aldrich), and endothelial cell growth supplement (5 µg/ml, made from calf brain) (complete culture medium). Culture medium for HUVEC was M199 (GIBCO BRL) with the same supplements.

RNAi

RNAi of DEP-1 expression was induced with siRNA directed against DEP. Positive 21-nucleotide siRNA (GGG.CCA.GGU.CCU.GUG.CGC.A.dT.dT, and U.GGG.AC.CGA.CCA.GGU.CCC.dT.dT) targeted murine DEP-1 sequence 86 nucleotides downstream of the start codon. As a negative control, the Scramble II Duplex, sAICE-RNAi, was used. Both positive and control oligonucleotides were from Dharmacon Research.

VE-cadherin-positive cells were seeded at a density of 20,000/cm² the day before transfection, and they were ~40% confluent at the time of transfection with 100 nM positive or scramble oligonucleotides in Oligofectamine (Invitrogen) and OptiMEM (GIBCO BRL), without serum or BSA. Before transfection, cells were washed once with OptiMEM. Transfection medium was maintained on cells for 4 h. Then it was removed and cells were cultured in complete medium. Transfection was repeated every 24 h for 2 d, and cells were used for the experiment after another 24 h. DEP expression was tested by Western blot as described above.

Proliferation assay

Cells (50,000 cell/cm²) were cultured for 48 h in culture medium (1 ml) on fibronectin-coated (7 µg/ml; Sigma-Aldrich) glass coverslips set in a 24-well plate. Cell layers were washed once in MCDB 131 (GIBCO BRL) and cultured for another 24 h in MCDB 131 with 1% BSA, with a wash and change of medium after 24 h. Fresh MCDB 131 with 1% BSA was then added containing VEGF (80 ng/ml; PeproTech) when indicated. Cells were confluent during the stimulation (~100,000 cell/cm²).
When subconfluent cultures were tested, cells at seeding were 5,000 cell/cm², which reached a density of ~20,000 cell/cm² at the time of VEGF stimulation. At 20 h from the beginning of the stimulation, BrdU (30 μM) was added, and the incubation continued for another 4 h. Cells were then fixed with 3% paraformaldehyde and treated for 10 min with 2 N HCl. BrdU incorporation into nuclear structures was put in evidence with anti-BrdU antibodies (mouse monoclonal, Amersham Biosciences), followed by TRITC-conjugated antibody to mouse immunoglobulin (DakoCytomab). BrdU-positive cells were counted in random fields up to a number of 300 total nuclei/sample.

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