Platelet Endothelial Cell Adhesion Molecule-1 and Vascular Endothelial Cadherin Cooperatively Regulate Fibroblast Growth Factor-induced Modulations of Adherens Junction Functions

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Cellular adherens junctions are formed by cadherins linked to proteins of the catenin family. In endothelial cells, not only vascular endothelial cadherin but also platelet endothelial cell adhesion molecule-1 localizes into junctions and associates with β-catenin. To explore a putative cooperation of platelet endothelial cell adhesion molecule-1 and vascular endothelial cadherin, we analyzed transfectants expressing either platelet endothelial cell adhesion (CD31 cells) or vascular endothelial cadherin (CD144 cells) or both molecules (CD31/CD144 cells), and, for comparison, human umbilical vein endothelial cells. Basic fibroblast growth factor completely dissociated vascular endothelial cadherin/β-catenin complexes and robustly moved β-catenin into the nucleus in CD144 cells, whereas in CD31/CD144 cells as well as in human umbilical vein endothelial cells, fibroblast growth factor only partially dissociated the junctional complex followed by a significantly reduced nuclear translocation of β-catenin. In contrast, in CD31 cells, the subcellular distribution of β-catenin remained unaffected by fibroblast growth factor. As a functional consequence, fibroblast growth factor induced a complete collapse of the F-actin network in CD144 cells, a limited rearrangement of F-actin fibers in CD31/CD144 cells and no F-actin rearrangement in CD31 cells. We also analyzed the effect of fibroblast growth factor-induced rearrangement of junctions on junction permeability for leukocytes: in line with our observation that vascular endothelial cadherin was required for cells to respond to fibroblast growth factor, only in CD31/CD144 cells, but not in CD31 cells, leukocyte transmigration was significantly enhanced by fibroblast growth factor. In conclusion platelet endothelial cell adhesion molecule-1 cooperates with vascular endothelial cadherin in a mutual fashion; platelet endothelial cell adhesion molecule-1 reduces and temporarily limits fibroblast growth factor-induced dissociation of vascular endothelial cadherin/β-catenin complexes, but requires vascular endothelial cadherin to control leukocyte transmigration in dependence of fibroblast growth factor. Key words: actin/β-catenin/CD31/CD144/transmigration. J Invest Dermatol 116:110–117, 2001

Several in vivo and in vitro studies have elaborated a role for platelet endothelial cell adhesion molecule-1 (PECAM-1, also called CD31) in vessel formation and leukocyte migration through vascular barriers (Albelda et al, 1990; Tanaka et al, 1992; Berman and Muller, 1995; DeLisser et al, 1997; Matsumura et al, 1997; Pellagatta et al, 1998). PECAM-1 is a 130 kDa transmembrane molecule of the immunoglobulin gene superfamily constitutively expressed on all endothelial cells. The extracellular domain of PECAM-1 contains six immunoglobulin-like homology domains, which support cell–cell adhesion by interacting with homotypic as well as heterotypic ligands (Muller et al, 1989; Piali et al, 1995; Prager et al, 1996). The cytoplasmic tail of PECAM-1 contains numerous potential sites for the phosphorylation of serine, threonine, and tyrosine residues (Newman et al, 1990; Lu et al, 1997). Upon phosphorylation of the tyrosine residues Y-663 and Y-686, PECAM-1 associates with protein tyrosine phosphatases SHP-1 and SHP-2 (Jackson et al, 1997; Hua et al, 1998; Newton-Nash and Newman, 1999) as well as the phospholipase C-γ1 and the inositol 5′-phosphatase SHIP (Pumphrey et al, 1999); however, it was only partially possible to match the downstream signal transduction of PECAM-1 with biological functions of PECAM-1 (Poggi et al, 1996; Jackson et al, 1997; Pellagatta et al, 1998; Newton-Nash and Newman 1999). Notwithstanding the considerable body of evidence that PECAM-1 is a potent signaling molecule and important for vessel formation and leukocyte migration, a PECAM-1-deficient mouse had a surprisingly mild phenotype; the vascular development was normal and leukocyte transmigration was only mildly affected (Duncan et al, 1999), indicating that PECAM-1 exerts its function in...
conjunction with other molecules, which—at least partially—can compensate for the absence of PECAM-1.

The other constitutively expressed transmembrane adhesion molecule implicated in the regulation of endothelial tube formation during vasculogenesis and angiogenesis as well as in mechanisms influencing leukocyte transmigration is vascular endothelial cadherin (VE-cadherin, also called cadherin-5 or CD144) (Del Maschio et al, 1996; Allport et al, 1997; Vittet et al, 1997). In contrast to the situation seen with PECAM-1, a VE-cadherin knockout mouse is unable to organize endothelial cells into vessel-like patterns (Vittet et al, 1997), indicating that VE-cadherin is irreplaceable for vessel formation. The expression of this 130 kDa protein is restricted to endothelial adherens junctions. The extracellular domain mediates homotypic cell contacts between adjacent endothelial cells (Lampugnani et al, 1992) and the cytoplasmic tail forms a complex with proteins of the catenin family and the F-actin-based cytoskeleton. Many lines of evidence suggest that catenins critically interfere with VE-cadherin function and vice versa. Cadherin-mediated adhesion progresses from a weak to a strong state, which depends on cadherin clustering and subsequent linkage to catenins and the cytoskeleton (Navarro et al, 1995; Adams et al, 1998; Yap et al, 1998; Aono et al, 1999). On the other hand, cadherin binding to β-catenin reduces the pool of β-catenin available for nuclear translocation (Fagotto et al, 1996; Torres et al, 1997; Ortenloff et al, 1997), whereas β-catenin interacts with and modulates the activity of a Le/Fc family of transcription factors (Behrens et al, 1996; Shuttman et al, 1999; Mann et al, 1999). The crucial importance of the interaction of VE-cadherin with β-catenin is substantiated by the finding that not only VE-cadherin knockout mouse, but also mice expressing a cytoplasmic truncation mutant of VE-cadherin, which fails to interact with β-catenin, have impaired vascular development and maturation (Carmeliet et al, 1999).

As outlined above, signaling properties of PECAM-1 and VE-cadherin are largely independent and yet there are no data available that describe an interference of PECAM-1 with VE-cadherin-dependent functions; however, based on light and electron microscopic studies showing that PECAM-1 colocalizes with VE-cadherin into junctional regions (Leach et al, 1993; Ayalon et al, 1999) and based on immunoprecipitation studies showing that PECAM-1 coprecipitates β-catenin (Matsumura et al, 1997; Ilan et al, 1999), we wished to explore if PECAM-1 operates as a regulator of adherens junction function.

MATERIALS AND METHODS

Cells and reagents
ECV304 cells (ATCC, Rockville, MD) were transfected with either PECAM-1 (CD31) or with VE-cadherin (CD144) cells or with cDNA for both proteins (CD31–CD144 cells) and subcultured as described previously (Halama et al, 1999). For each of the three transfectants, a set of at least four different single cell-derived clones was used for all following experiments. HUVEC were isolated and subcultured as described (Matsumura et al, 1997).

Preparation of full cell extracts for immunoprecipitation and western blotting
Cells (5×10^6) were lysed in 1 ml lysis buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM CaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg aprotinin per ml, 15 µg leupeptin per ml, 1% nonidet P-40 and 1% Triton X-100), homogenized with a motor-driven Teflon-type Elvehjem-Potter (Kontes, Vineland, NJ), incubated on ice for 1 h and centrifuged at 10,000×g for 30 min at 4°C. The supernatants were used for immunoprecipitation and subsequent immunoblotting as described (Matsumura et al, 1997). The monoclonal antibodies used were: anti-VE-cadherin (TEA1/31, Immunotech, Marseilles, France), anti-VE-cadherin–biotin (polyclonal rabbit antibody, BendermedSystems, Vienna Austria), anti-PECAM-1 (7E4) (Matsumura et al, 1997), anti-PECAM-1–biotin (158-2B3, Ancell Europe, La Èufelingen, CH), anti-β-catenin (clone 14), anti-plakoglobin (clone 15, both IP-10 (10 ng per ml, R&D, Minneapolis, MN). After confluence, cells were kept in culture for an additional 24 h. Then, peripheral blood mononuclear cells were prepared by density gradient centrifugation and loaded with CellTracker GreenM (Molecular Probes). Cells (10^5 per well) were seeded on top of the transfectants in the presence of 6 µl per ml anti-basic FGF (polyclonal rabbit antibody, Stratmann, Hanover, Germany) or 6 µl per ml rabbit serum (control) in RPMI (Life Technology). Cells were allowed to adhere and to transmigrate for 20 h. Adherent and transmigrated cells were imaged by confocal laser scan microscopy and analyzed by an automated computer program as described (Gröger et al, 1999).

RESULTS

The association of PECAM-1 with β-catenin requires VE-cadherin expression
We have selected the bladder–carcinoitally derived cell line ECV304 for transfection, because they lack expression of epithelial or endothelial cadherins or of PECAM-1, but express α-, β-, and γ-catenin (plakoglobin) (Halama et al, 1999). In CD31–CD144 cells, VE-cadherin as well as PECAM-1 coprecipitated β-catenin (Fig 1). Also the reverse experiment, precipitation with β-catenin, coprecipitated PECAM-1 as well as VE-cadherin in these cells (data not shown). As VE-cadherin and PECAM-1 did not coprecipitate (Fig 1), they most likely associate with separate β-catenin molecules. γ-catenin coprecipitated with VE-cadherin only but not with PECAM-1 (Fig 1). Thus, regarding to the composition of junctional protein complexes, our CD31–CD144 cells resembled human umbilical vein endothelial cells (HUVEC), where β-catenin associates with VE-cadherin as well as with PECAM-1 (Matsumura et al, 1997; Ilan et al, 1999) and γ-catenin with VE-cadherin only (Kowalczyk et al, 1998). Also in CD144 cells, VE-cadherin coprecipitated β-catenin as well as γ-catenin. By contrast, in CD31 cells, PECAM-1 was unable to coprecipitate any catenin (Halama et al, 1999).

FGF-induced dissociation and subsequent reformation of junctional complexes
In CD31–CD144 cells, basic FGF (20 ng per ml plus 5 U per ml heparin) induced a partial dissociation of VE-cadherin/β-catenin complexes, which maximized between 8...
and 24 h following FGF stimulation (minus 60% from baseline, Fig 2). Within 4 h following FGF withdrawal, VE-cadherin/β-catenin complexes had completely reassembled to the extent seen at baseline. Most interestingly, PECAM-1/β-catenin complexes remained stable even in the presence of FGF (Fig 2). HUVEC, analyzed for comparison, had similar kinetics of VE-cadherin/β-catenin complex dissociation in response to FGF (Fig 2). In striking contrast, in CD144 cells, FGF completely disrupted VE-cadherin/β-catenin complexes and reassembly of complexes was not completed before 48 h after FGF withdrawal (Fig 2). In none of the cell types there was a change in the total amount of VE-cadherin (Fig 3) and/or of PECAM-1 (not shown) during the time course of the experiment.

Laser scan images supported the results obtained by analyzing cell extracts (Fig 3). Following FGF stimulation, a translocation of β-catenin from the membrane into the cytosol and into the nucleus was only seen in CD31/CD144 cells and CD144 cells, but not in CD31 cells (Fig 3).

FGF-induced polarization of F-actin CD31–CD144 cells as well as CD144 cells produced a cobblestone-like growth pattern in cell culture identical to that seen with HUVEC (Matsumura et al, 1997; Halama et al, 1999). F-actin fibers were arranged in parallel bundles reaching from one cell border to the other and cells migrated as a strict monolayer (Fig 5). Following FGF stimulation, cells released their cell contacts and F-actin condensed in small protrusions at sites that had lost lateral cell–cell contact (Fig 5; arrows). Most importantly, in CD144 cells almost all the F-actin condensed into these protrusions (Fig 5 arrows; mean numbers of protrusions/cell were 4.5±0.8; results from four independent experiments), whereas in CD31–CD144 cells, considerable amounts of F-actin fibers remained stretched even in the presence of FGF and numbers of protrusions per cell were significantly reduced compared with CD144 cells (mean 1.4±0.2, p<0.001). It should be noted that the FGF-induced polarization of F-actin was completely blocked by cycloheximide (2 µg per ml; data not shown), indicating that FGF-induced polarization of F-actin depended on de novo protein synthesis.

Following FGF withdrawal, in CD31–CD144 cells, a realignment of F-actin fibers was seen within 24 h. In contrast, at this time point F-actin was still diffusely distributed throughout the cytoplasm in CD144 cells and a realignment of F-actin fibers was not detectable until 48 h following FGF withdrawal (Fig 5).

CD31 cells were considerably smaller than CD144 or CD31–CD144 cells, grew in polylayers and β-catenin was not targeted to junctional areas (Halama et al, 1999). Most importantly, F-actin was diffusely distributed throughout the cytosol at baseline (Fig 5) and FGF was unable to induce a polarization of F-actin as FGF did not induce a release of cell–cell contacts (Fig 5). It should be noted that Ilan et al (1999) transfected PECAM-1 into SW480 cells, which resulted in a localization of β-catenin to the cell membrane even in the absence of VE-cadherin. This discrepancy with our CD31 cells might be due to the fact that SW480 cells express a mutant adenomatosis polyposis coli protein, which causes constitutively elevated levels of free cytosolic β-catenin (Simcha et al, 1998).

Transmigration is most efficient in PECAM-1/VE-cadherin double transfectants and is inhibited by anti-FGF antibodies To test the functional consequences of PECAM-1 on the permeability of VE-cadherin-containing junctions for leukocyte transmigration, a semiautomated migration assay (Gröger et al, 1999) was employed with some modifications. To ensure vigorous leukocyte transmigration across ECV304 cells, a chemotactic gradient was created by incorporating a chemokine cocktail in the gel (see Materials and Methods). Under these conditions, CD144 cells were rather impermeable for leukocytes with the adhesion/migration ratio being approximately 20:1 (Fig 6). CD31 cells were more permeable for leukocyte transmigration (Fig 6), the adhesion/migration ratio being in the range of 10:1 (p<0.05). Given the large numbers of reports detailing the
importance of PECAM-1 in leukocyte transmigration (Berman and Muller, 1995; Piali et al., 1995; Poggi et al., 1996; Prager et al., 1996; Pellegatta et al., 1998), we attributed the leukocyte transmigration seen across CD31 cells mainly to PECAM-1 interactions with ligands expressed on the leukocytes rather than being dependent on reduced junction adhesiveness.

Most interestingly, CD31−CD144 cells were highly permeable for leukocytes, the adhesion/migration ratio being 2.5 : 1 (Fig 6; p<0.05 as compared with CD31 cells and CD144 cells), which was well within the range of the transmigration efficacy when unstimulated HUVEC were used in this modified migration assay (Fig 6). In view of the experiments described in Figs 2, 3, and 5 showing that VE-cadherin/PECAM-1 coexpression enhances junction stability, this enhanced transmigration seen across CD31−CD144 cells was unexpected. As monocytes are a rich source of FGF (Arras et al., 1998) and as the adhesive strength of PECAM-1/VE-cadherin-containing junctional regions is regulated by FGF, hepatocyte growth factor, epidermal growth factor, or thrombin (Hoschuetzky et al., 1994; Rabiet et al., 1996; Hazan and Norton, 1998; Hiscox, 1999).

Our initial experiments showed that PECAM-1 reduced and temporarily limited the FGF-induced dissociation of VE-cadherin/β-catenin complexes. This stabilizing effect of PECAM-1 is most likely mediated indirectly, because PECAM-1 and VE-cadherin do not physically interact. As the cytoplasmic tails of PECAM-1 and VE-cadherin have no homology, they likely use distinct region on β-catenin for binding. Signals regulating the stability of the VE-cadherin/β-catenin complexes are only partially understood. The association of protein-tyrosine phosphatases such as PTPmu, PTP-1B, or LAR-PTP with the VE-cadherin/β-catenin complex have been shown to increase the cohesivity of the complex (Kypta et al., 1996; Balsamo et al., 1998; Brady-Kalnay et al., 1998; Muller et al., 1999). As also PECAM-1 can associate with protein-tyrosine phosphatases via its src homology 2 domain binding motif (Jackson et al., 1997; Hua et al., 1998; Newton-Nash and Newman, 1999), this may interfere with the stability of the cadherin/β-catenin complexes, but the design of our experiments did not address this question.

Following FGF stimulation, the nuclear translocation of β-catenin is 2–3-fold less in CD31−CD144 cells as compared with CD144 cells. The simplest explanation for this effect is based on our observation that PECAM-1 stabilizes VE-cadherin/β-catenin complexes, which results in a quantitative reduction of β-catenin available for nuclear translocation. Although the source of nuclear

**DISCUSSION**

PECAM-1 and VE-cadherin participate in several interrelated endothelial functions such as vessel formation and leukocyte transmigration, but it is unknown whether VE-cadherin interferes with PECAM-1-dependent functions and vice versa. It is well documented that cadherin/β-catenin complexes control cell adhesion and transfer signals to and between cells. The interactions between cadherins and catenins are dynamic and regulated by FGF, hepatocyte growth factor, epidermal growth factor, or thrombin (Hoschuetzky et al., 1994; Rabiet et al., 1996; Hazan and Norton, 1998; Hiscox, 1999).

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β-catenin is still under debate, there are many lines of evidence that the binding of β-catenin to junctional regions antagonizes the signaling properties of β-catenin (Fagotto et al., 1996; Torres et al., 1996; Papkoff, 1997; Orsulic et al., 1999). We show that FGF did not alter the total cellular amount of β-catenin, but reduced the junctional pools and subsequently increased the cytosolic and the nuclear pools of β-catenin. Moreover, in CD31 cells, where PECAM-1 was unable to bind and localize β-catenin to the cell membrane (Halama et al., 1999), FGF was unable to alter the subcellular distribution of β-catenin (Figs 3 and 4), which supports the view that FGF translocated junctional catenin into the nucleus. The situation is complicated by the fact that the steady-state levels of cytosolic β-catenin levels are not only controlled by transmembrane proteins, but also by several interacting proteins within the cytosol, including, e.g., axin, adenomatosis polyposis coli protein, and the protein kinase GSK-3β (Papkoff et al., 1996; Hart et al., 1998; Ikeda et al., 1998; Willert et al., 1999); however, their role in regulating β-catenin activity and stability has been largely investigated in response to Wnt but not to FGF signals. Wnt-1-induced nuclear translocation of β-catenin has recently been found to also function in human endothelial cells (Wright et al., 1999), but it remains yet unresolved, whether the FGF-induced nuclear translocation of β-catenin, which in our system requires junctional β-catenin, depends on or bypasses cytosolic regulatory proteins of β-catenin activity. An adenomatosis polyposis coli protein-independent regulation of β-catenin activity has been described in colon carcinoma cell lines following phorbol myristate acetate stimulation (Baulida et al., 1999).
We (Halama et al 1999) and others (Breviario et al, 1995; Caveda et al, 1996) have previously shown that transfection of VE-cadherin alone, even in the absence of PECAM-1, is sufficient to mediate monolayer formation, cell contact-dependent growth arrest and tube formation irrespective of the cell type used for transfection. Under unstimulated conditions, such transfected cells mimic growth behavior of real endothelial cells (Matsumura et al, 1997), indicating that this is independent from PECAM-1. The situation is different following FGF stimulation. Whereas CD144 cells almost completely lost their cell-cell contacts and most of the actin condensed at the protruding edges of the cell, in CD31-CD144 cells the FGF-induced loss of cell contact and the collapse of F-actin fibers was significantly reduced and comparable with that seen in HUVEC. Again, this finding is in agreement with our initial
observation that VE-cadherin/β-catenin complexes were only partially dissociated by FGF in CD31–CD144 cells, thereby allowing a limited linkage of F-actin to the junction. In contrast, in CD144 cells, VE-cadherin/β-catenin complexes were completely disrupted by FGF, resulting in the total collapse of F-actin fibers. It should be noted that mock-transfected cells (data not shown), just like CD31 cells, were unable to respond to FGF stimulation.

To characterize further the functional consequences of FGF-induced rearrangement of junctions, we performed leukocyte transmigration studies. With regard to CD31 cells, it is thoroughly documented that PECAM-1 molecules “track” leukocytes through junctions (Berman and Muller, 1995; Piali et al, 1995; Poggi et al, 1996; Prager et al, 1996; Pellegatta et al, 1998). Thus, CD31 cells allowed a certain amount of leukocyte transmigration, but this was not regulated by FGF, inasmuch as FGF was unable to alter the distribution of β-catenin or F-actin fiber rearrangement. CD144 cells did not support leukocyte transmigration, whether FGF was present or not, which we attributed to the fact that ECV-304 cells lack molecules to “track” leukocytes to the junctions. The most intriguing finding is that FGF enhanced leukocyte transmigration in CD31–CD144 cells and in HUVEC, which correlates with our observation that FGF opened junctional areas in CD31–CD144 cells and in HUVEC. Our experiments confirm previous reports that the dissociation of VE-cadherin/β-catenin complexes plays an important part in leukocyte transmigration (Del Maschio et al, 1996; Allport et al, 1997). We extend this knowledge in several ways: (i) in addition to leukocyte adhesion-induced dissociation of the adherens junction complex, also a soluble factor, FGF, dissociates these complexes and supports transmigration; (ii) PECAM-1–dependent mechanisms of directing the leukocyte through junctions are regulated by VE-cadherin; and (iii) dissociation of junctions is not sufficient to allow transmigration as indicated by the spoiled transmigration in FGF-stimulated CD144 cells. In addition to junction dissociation, transmigration depends on molecules that “track” leukocytes through the junction, which in our experimental system is PECAM-1; however, it should be noted that in vivo this function of PECAM-1 appears to be redundant (Duncan et al, 1999).

In conclusion, we describe a novel functional cooperation between PECAM-1 and VE-cadherin. The biologic relevance of results obtained with our transfection system are validated by the fact that HUVEC behave like the double transfecants in all assays employed. We demonstrated that VE-cadherin is required for FGF to induce nuclear translocation of β-catenin, F-actin rearrangement, and cell scattering as well as for FGF to open and close PECAM-1–dependent pathways of leukocyte transmigration. Most importantly, we have shown that PECAM-1 operates as a regulatory component of VE-cadherin-containing adherens junctions. PECAM-1 reduces and temporally limits FGF-induced dissociation of VE-cadherin/β-catenin complexes and allows for their rapid reassembly. This function appears to be particularly important for efficient and fast restoration of the integrity of endothelial linings following junction dissociation, a condition given in vivo, e.g., in wound healing or in inflammation.

This work was supported by grants from the Austrian Science Foundation (P12240-MED and SFB005) and from the Naichos Foundation.

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