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PECAM-1 promotes β-catenin accumulation and stimulates endothelial cell proliferation☆

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Abstract

Platelet endothelial cell adhesion molecule-1 (PECAM-1) binds tyrosine phosphorylated β-catenin and modulates β-catenin localization [J. Immunol. 158 (7) (1997) 3408; J. Cell Sci. 112 (Pt 18) (1999) 3005]. To elucidate functional consequences of this interaction, we studied endothelial cells from PECAM-1 knockout animals and compared them to PECAM-1 expressing endothelial cells [Mol. Biol. Cell 11 (9) (2000) 3109]. We noted an increase in the expression of β-catenin protein in PECAM-1 expressing endothelial cells. Further, by immunofluorescence, β-catenin localized to the cell membrane as well as to the nucleus in PECAM-1 positive endothelial cells, whereas cells not expressing PECAM-1 stained for β-catenin only at the membrane. Additionally, we demonstrate that PECAM-1 lacking the majority of the cytoplasmic domain promotes significantly less accumulation of transcriptionally active β-catenin than full-length PECAM-1. Finally, we note an increased proliferative rate in the PECAM-1 reconstituted cells compared to the endothelial cells lacking PECAM-1. Taken together, our data suggest that PECAM-1, an adhesion molecule, affects cell proliferation via accumulation of transcriptionally active β-catenin.

Keywords: PECAM-1; β-Catenin; Proliferation; Endothelium

Platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) is expressed exclusively on endothelial cells, platelets, and specific cells of the immune system [26]. Its ectodomain mediates adhesion [3], while its cytoplasmic portion serves as a scaffold for signaling [14,32] and cytoskeletal proteins [15,16]. PECAM-1 is an important modulator of adhesion [5], cell migration [8,35], inflammatory cell infiltration [6], brain endothelial barrier permeability [11], endothelial permeability [19], bleeding time [20], angiogenesis [3,8], phagocytosis of macrophages [7], and endothelial cell apoptosis [17].

An important cytosolic binding partner of PECAM-1 is β-catenin [21]. β-Catenin is a component of adherens junctions and mediates adhesion by providing a physical link between the transmembrane protein E-cadherin and the actin cytoskeleton [33] via interactions with α-catenin [2]. Tyrosine phosphorylation of β-catenin disrupts the interaction between E-cadherin and β-catenin, thus enabling β-catenin to localize to the cytosol [18,37]. Levels of free β-catenin are strictly controlled [37] by either degradation via the ubiquitin–proteasome system [1] or by tyrosine phosphorylation [28,29] and stabilization [30]. Free β-catenin can translocate to the nucleus to activate the transcription of growth regulating genes such as cyclin D1 and c-myc [12,36,40].

β-Catenin also plays a pivotal role in the Wnt/Wingless growth factor signaling pathway. Wnt signaling events are initiated by receptor activation involving the frizzled cell surface protein (reviewed in [22]). This signal suppresses the activity of glycogen synthase kinase 3 (GSK-3) by serine phosphorylation, leading to increased stability of β-catenin. β-Catenin can then complex with members of the *Drosophila* Tcf family transcription factors, regulating the expression of the Wnt target genes [13].

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PECAM-1 binds tyrosine phosphorylated β-catenin and this dynamic interaction is involved in angiogenesis [16]. Evidence from transfection experiments suggests that PECAM-1 may function to localize and sequester β-catenin at the cell membrane [16]. Our present study demonstrates that expression of PECAM-1 promotes the accumulation of β-catenin that is transcriptionally active and identifies PECAM-1 as a modulator of cell proliferation. We propose that PECAM-1 affects cell proliferation by participating in the Wnt signaling pathway, thereby stabilizing β-catenin.

Materials and methods

Cells and cell culture. Lung endothelial cells from PECAM-1 knockout mice (Lu−) were transfected with full-length PECAM-1 (Lu+) and colonies were selected and maintained in puromycin (kind gift of Dr. Britta Engelhardt, Max-Planck Institute, Munster, Germany), and cultured as described [39]. Lu− and Lu+ cells were used between passages 5 and 30. 293 fibroblasts were obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in DMEM with 10% FBS.

Cell lysate preparation, immunoprecipitation, and Western blotting. Confluent monolayers were washed in ice-cold PBS, lysed in buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% deoxycholate, 10% glycerol, and 150 mM NaCl with Complete protease inhibitor cocktail (Boehringer Mannheim), and 1 mM sodium orthovanadate), and centrifuged for 20 min at 12,000 rpm at 4 °C and protein assay on supernatants was done with BCA Protein assay kit (Pierce, Rockford, IL) using BSA as standard.

Five hundred micrograms of protein was immunoprecipitated with anti-PECAM-1 (Santa Cruz Laboratories) or β-catenin (Transduction Labs, San Diego, CA) antibody in immunoprecipitation (IP) buffer (50 mM Tris, pH 7.4, 5 mM EDTA, pH 7.4, 150 mM NaCl, and 0.5% NP-40) with protein AG Sepharose (Santa Cruz Technologies, Santa Cruz, CA). IPs were incubated overnight at 4 °C, centrifuged at 12,000 rpm at 4 °C and washed three times with IP buffer, then solubilized in 2× SDS sample buffer, boiled at 100 °C for 10 min, and loaded onto 8% SDS gels. Western blotting was done as described [1]. The blots were stripped and reprobed with ERK2 to normalize for protein loading.

Immunfluorescence. Lu− and Lu+ cells were washed with PBS, fixed with 4% paraformaldehyde, washed with PBSA (PBS with 1% bovine serum albumin), permeabilized with 0.2% Triton X-100 for 30 min, followed by incubation in blocking buffer (1:40 normal donkey serum in PBS) for 30 min, and then incubated in primary antibodies (Sleet 4-rabbit polyclonal anti-mouse CD31 and monoclonal anti-β-catenin in PBS) for 2 h at room temperature. After washing with PBSA, secondary antibodies were added (donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa fluor 594) and incubated for 30 min. After washing, DAPI (10 μg/ml) was added for 10 min. Specimens were washed, mounted using PVA-glycerol, and viewed using a Zeiss Research fluorescence microscope. Micrographs were taken with a SPOT digital camera using SPOT Software (Diagnostic Instruments, Sterling Heights, MI) and a Macintosh G4 computer running Photoshop 5.0 software (Adobe Systems, San Jose, CA).

β-Catenin reporter assays. TOPFlash is a reporter plasmid containing two sets of three copies of the T-cell factor (TCF) binding site upstream of the thymidine kinase (TK) minimal promoter and luciferase open reading frame. FOPFlash containing mutated TCF binding sites serves as a negative control. 293 cells were transfected with 50 ng β-gal, 100 ng TOPFlash or FOPFlash (Upstate Biotechnology), and 3 μg of either pcDNA, full-length PECAM-1 or Δ11–16 PECAM-1 (exons 11–16 deleted, kind gifts of Dr. Steven Albelda, University of Pennsylvania). Lipofectamine 2000 (Invitrogen) was used to transfect cells in Optimem (GIBCO BRL) for 3 h. Forty-eight hours after transfection, cells were lysed in Reporter Lysis Buffer (Promega), assayed for luciferase activity using Promega's Luciferase Assay kit and β-galactosidase activity using Tropix's Galacto-Light kit, both as per manufacturer's instructions, and analyzed on a Wallac Victor^2 1420 Multilabel Counter. Transfections were done in duplicates and four sets of experiments were performed with similar results.

Proliferation assay. Proliferation was assessed by cell counts, Western blot analysis for PCNA, and FACS analysis following BrdU incorporation. Lu− and Lu+ cells were plated at identical densities and visualized under the microscope 24 h after plating to determine equal adhesion in both samples. There were no floating cells in either specimen. Lu− and Lu+ cells were counted by trypan blue exclusion using a hemocytometer 3 and 6 days after plating. Western blotting for PCNA was done to correlate with the observed differences in proliferation. To confirm that the differences in proliferation between the Lu− and Lu+ cells were attributable to PECAM-1, 293 cells were transfected with pcDNA or PECAM-1 as described above. Twenty-four hours after transfection, 25 μg/ml BrdU was added and cells were incubated for 16 h after which they were trypsinized and washed with PBSA. Staining was done as per Pharmingen's instructions using 20 μl BrdU-FITC (Sigma) and 20 μl Mec 13.3-PE (Transduction Labs). After centrifugation, the cells were resuspended in PBSA/0.5%Tween 20 plus 20 μl BrdU-FITC (Sigma) and 20 μl Mec 13.3-PE (Transduction Labs) and incubated at room temperature for 30 min. Cells were washed with PBSA followed by analysis on a BD FACScan using CellQuest software. Data are representative of two independent experiments each performed in triplicate.

Results

Increased pool of β-catenin upon PECAM-1 expression

Prior studies indicate that transfection of PECAM-1 into SW480 cells, which do not endogenously express PECAM-1, led to localization of β-catenin at the cell membrane while decreasing the amount of β-catenin in the cytoplasm [16]. Since sequestration of β-catenin at the membrane by PECAM-1 might affect total amounts of β-catenin, we examined the expression levels of β-catenin in Lu− and Lu+ cells. Western blot analysis of Lu− and Lu+ cell lysates revealed that Lu+ cells express twice the amount of β-catenin compared to the Lu− cells (Fig. 1A) ($p < 0.05$). IPs of β-catenin from the same lysates were blotted with anti-phosphotyrosine, then stripped, and reprobed with anti-β-catenin to obtain the fraction of tyrosine phosphorylated β-catenin. Only, 10% of the increase in β-catenin in Lu+ cells can be accounted for by the tyrosine phosphorylated pool of β-catenin while 90% of the increase is in the non-tyrosine phosphorylated fraction of β-catenin.

To verify that expression levels of β-catenin were not due to transfection or selection of Lu+ cells, we transfected full-length PECAM-1 or GFP vector into 293 cells that do not express endogenous PECAM-1. 293 cells transfected with GFP vector were used as controls and to estimate the transfection efficiency. Transfection

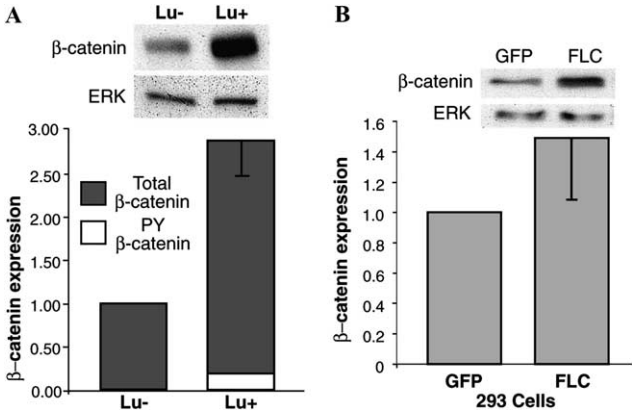

Fig. 1. (A) Lu− and Lu+ lysates were Western blotted for β-catenin and normalized with ERK-2. Lysates of Lu− and Lu+ cells were immunoprecipitated for β-catenin followed by Western blotting with anti-phosphotyrosine antibody. The bars represent amounts of β-catenin normalized for ERK-2. The unshaded box in Lu+ cells represents the fraction of tyrosine phosphorylated β-catenin. Lu− cells have no detectable tyrosine phosphorylated β-catenin. The experiment was performed three times and results are expressed as means ± SE. (B) 293 cells were transfected with GFP or PECAM-1 and lysates were blotted with β-catenin and normalized with ERK-2. Transfections were repeated three times and results are expressed as means ± SE.

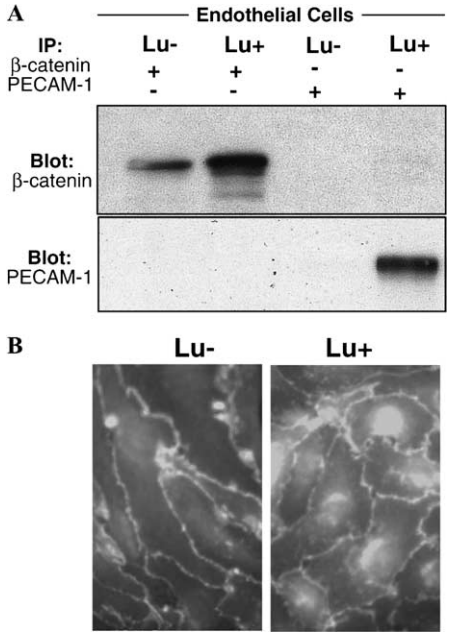

Fig. 2. (A) Lysates of Lu− and Lu+ cells were IPed for β-catenin and probed for PECAM-1. Conversely, Lu− and Lu+ lysates were IPed for PECAM-1 and probed for β-catenin. Negligible coprecipitation of β-catenin with PECAM-1 was noted. IPs were repeated at least three times. Illustrated is a representative experiment. (B) Confluent layers of Lu− and Lu+ cells were stained with β-catenin and viewed using a fluorescence microscope. The figure shows β-catenin staining cell membranes in Lu− cells, while Lu+ cells have β-catenin staining in cell membranes as well as nuclei.

of PECAM-1 into 293 fibroblasts increased the expression of β-catenin by 40% (Fig. 1B) ($p < 0.05$). The apparent discrepancy in fold induction of β-catenin between Lu and 293 cells can be explained by the fact that the transfection efficiency of the 293 cells is approximately 70% (as judged by GFP expression) and the Western blot represents β-catenin expression levels of both transfected and non-transfected cells. These findings suggest that PECAM-1 induces an increase in β-catenin levels in endothelial cells and fibroblasts transfected with PECAM-1.

PECAM-1 affects localization of β-catenin in endothelial cells

Binding of β-catenin to PECAM-1 at the membrane may be a mechanism for sequestration, resulting in increased levels of β-catenin. Since only tyrosine phosphorylated β-catenin binds to PECAM-1, we should expect an increase in the pool of tyrosine phosphorylated β-catenin and an increase in the amount of β-catenin that coprecipitates with PECAM-1. However, we have already demonstrated that only 10% of the increased pool of β-catenin in Lu+ cells is tyrosine phosphorylated. This would imply that a very small fraction of β-catenin is bound to PECAM-1 in the Lu+ cells. IP of Lu− and Lu+ lysates with PECAM-1 antibody and Western blotting with anti-β-catenin confirms that a negligible amount of β-catenin is bound to PECAM-1 (Fig. 2A). Conversely, IP of β-catenin followed by Western blotting for PECAM-1 confirms that very little β-catenin is bound to PECAM-1. This suggests that the induction in β-catenin in PECAM-1 expressing cells cannot be attributed to the binding and sequestration of β-catenin to PECAM-1 at the cell membrane. PECAM-1 has been shown to constitutively coprecipitate with β-catenin in EOMAs pretreated with vanadate [16]. Vanadate leads to tyrosine phosphorylation of β-catenin and therefore increases its association with PECAM-1. In our experimental system, vanadate pretreatment was not used and would explain the lack of coprecipitation of PECAM-1 with β-catenin.

Immunofluorescence analyses of Lu− and Lu+ cells for β-catenin (Fig. 2B) demonstrated that in Lu− cells, β-catenin localized to the cell membrane, while in the Lu+ cells, β-catenin was found in cell membranes as well as in nuclei. β-Catenin has been noted to localize to the membrane in confluent cultures while cytoplasmic and nuclear distribution has been demonstrated in proliferating, subconfluent cultures [9]. Moreover, nuclear distribution of β-catenin has been correlated with cellular proliferation. Our finding of nuclear β-catenin staining in PECAM-1 expressing cells raised the intriguing possibility that expression of PECAM-1 might promote the accumulation of transcriptionally active β-catenin.

Transcriptional activation of β-catenin mediated by PECAM-1 expression

To determine whether the pool of PECAM-1 induced β-catenin is transcriptionally active, luciferase reporter assays on lysates of 293 cells transfected either with pcDNA, full-length PECAM-1 or Δ11–16 PECAM-1, plus β-gal and either TOPFlash or FOPFlash were performed. β-Gal activity was used to correct for transfection efficiency of all samples. TOPFlash or FOPFlash luciferase activity of samples transfected with pcDNA was subtracted from samples expressing full-length or Δ11–16 PECAM-1. Full-length PECAM-1 stimulated a sevenfold increase in TOPFlash luciferase activity compared to FOPFlash (Fig. 3A). In contrast, in 293 cells transfected with the Δ11–16 PECAM-1 construct, TOPFlash luciferase activity was increased only twofold over FOPFlash luciferase activity (Fig. 3B) ($p < 0.05$, full-length PECAM-1 vs. Δ11–16 PECAM-1). This suggests that full-length PECAM-1 promotes more accumulation of transcriptionally active β-catenin than does PECAM-1 lacking the majority of the cytoplasmic domain. This implies that while the ectodomain of PECAM-1 might be involved in modulation of β-catenin levels via the Wnt signaling pathway, the cytoplasmic region of PECAM-1 may transmit signals inhibiting degradation or increasing transcriptional activity of β-catenin. Alternatively, the cytoplasmic portion of PECAM-1 encoded by exon 10 is likely regulating the signaling pathway, leading to induction of β-catenin. This invokes the exciting possibility that an adhesion molecule such as PECAM-1 may be involved in cell signaling through β-catenin and affect important biological responses.

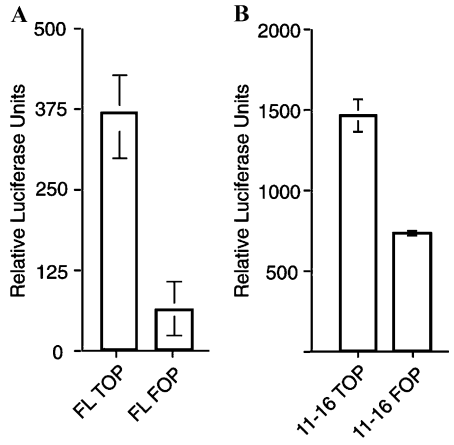

Fig. 3. 293 cells were transfected with Δ11–16 (B) or full-length PECAM-1 (A) and TOPFlash or FOPFlash and β-gal. (A) Demonstrates luciferase induction in transfectants expressing full-length PECAM-1 ($p < 0.05$ for TOPFlash vs. FOPFlash) while (B) demonstrates luciferase induction in Δ11–16 transfectants ($p < 0.05$ for TOPFLash vs. FOPFlash). Data are means of four experiments ± SE performed in duplicates and corrected for transfection efficiency using β-gal. Background TOPFlash and FOPFlash luciferase activity of pcDNA transfected cells was subtracted.

PECAM-1 induces cellular proliferation

Accumulation of transcriptionally active β-catenin is associated with proliferation of cells in various in vitro systems [9,23,38]. Since we had noted that cells transfected with PECAM-1 had accumulated transcriptionally active β-catenin, we reasoned that these cells might have higher proliferative capacities. Lu− and Lu+ cells grown in regular media were counted by trypan blue exclusion 3 and 6 days after plating. No significant difference was seen in the number of cells stained with trypan blue (data not shown) and there was no difference in adhesion as determined by visualizing the cells under the microscope. Lu+ cells had higher viable cell counts compared to the Lu− cells at both 3 and 6 days (Fig. 4A, left panel) ($p < 0.05$ Lu− vs. Lu+ at day 6). The higher cell number correlates well with the increased expression of PCNA noted by Western blotting (Fig.

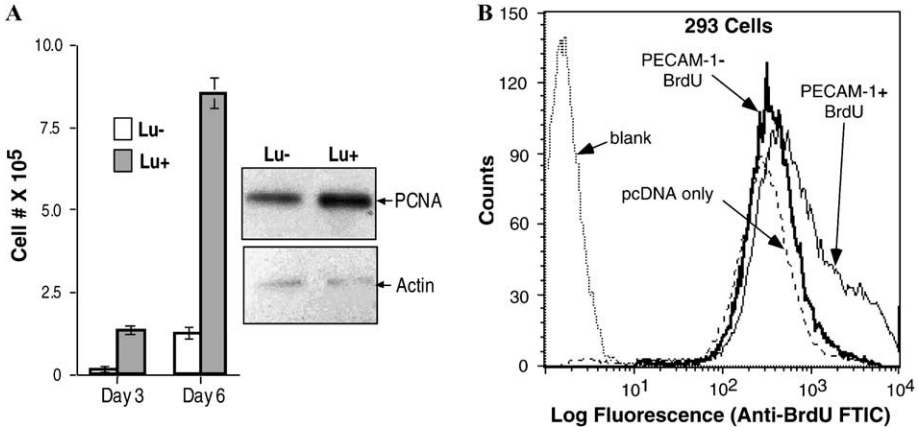

Fig. 4. (A) Lu− and Lu+ cells were counted using a hemocytometer 3 and 6 days after plating. Data are means ± SE of four separate experiments. (B) Representative FACS analysis of 293 cells transfected with pcDNA or PECAM-1, treated with BrdU for 16 h, and stained with FITC-anti-BrdU. The experiment was performed twice in triplicate with similar results.

4A, right panel). Although PECAM-1 has been demonstrated to be a survival factor, the lack of proliferation of the Lu− cells were not due to accelerated apoptosis of those cells as evidenced by the lack of an increase in trypan blue uptake.

Since the Lu− cells were transfected with PECAM-1 and subsequently selected with puromycin, we wanted to confirm that the differences in proliferation between the Lu− and Lu+ cells was not due to induction of growth promoting genes resulting from transfection and selection. We transfected 293 cells with PECAM-1 and performed BrdU incorporation. Fig. 4B demonstrates that 293 cells transfected with PECAM-1 had a 20% higher incorporation of BrdU, suggesting higher proliferative rates. This finding confirms a PECAM-1 induced increase in proliferative rate in Lu+ compared to Lu− cells. Moreover, given the ability of PECAM-1 to promote proliferation in both 293 cells (fibroblasts) and in endothelial cells, the signaling pathway utilized for this biological response is likely to be common to both cell types.

Discussion

In addition to their structural and functional roles in cell–cell junctions, PECAM-1 and β-catenin are important participants in intracellular signaling events. Increase in the free pools of β-catenin has been associated with epithelial cell migration and tumorigenesis [23,24]. In microvascular endothelial cells, expression of exogenous metabolically stable β-catenin stimulated growth, suggesting that the β-catenin signaling pathway was active in endothelial cells [38]. In endothelial cells expressing full-length PECAM-1, we note a doubling in β-catenin expression. This induction of β-catenin by PECAM-1 cannot be explained simply by membrane sequestration of β-catenin by PECAM-1.

β-Catenin localizes to the nucleus in endothelial cells upon PECAM-1 transfection. Conversely, in SW480 cells (human colon carcinoma), exogenous expression of PECAM-1 causes sequestration of β-catenin at the cell membrane and the effect of this translocation on proliferation remains unknown. At baseline, SW480s have defective APC protein, lack E-cadherin and PECAM-1, and accumulate β-catenin in the nucleus, correlating with a high proliferative rate. Lu− cells express lower baseline levels of β-catenin, predominantly at the cell membrane, and exogenous expression of PECAM-1 (Lu+ cells) results in an increase in total β-catenin, with a change in localization to the nucleus and a higher proliferative rate. Collectively, these data suggest that PECAM-1's function is contextual and varies with cell type. This would explain the ability of PECAM-1 to sequester β-catenin in cells with large amounts of endogenous β-catenin (SW480) while promoting the accumulation of β-catenin in cells with low baseline levels of this protein and an intact β-catenin degradation machinery (lung endothelial cells and 293 cells).

PECAM-1-β-catenin interactions play a role in the progression of angiogenesis in vitro [16]. Angiogenesis is composed of four tightly regulated processes; degradation of basement membrane, migration, proliferation, and differentiation. Our present study has elucidated that the interaction between PECAM-1 and β-catenin is involved in the proliferation phase of angiogenesis. This is supported by the observation that brain derived endotheliomas (EOMAs) [27] have high levels of PECAM-1 and β-catenin expression which correlates with their rapid proliferative rates. We speculate that upon cytokine stimulation, endothelial cells initially induce PECAM-1 followed by increased expression of β-catenin leading to transcriptional activation of growth promoting genes and thereby proliferation. Proliferation of endothelial cells upon induction of PECAM-1 and consequently β-catenin expression might be an alternative pathway in the regulation of endothelial cell growth. Endothelial cells derived from various microvascular beds might respond differentially to cytokines and therefore to PECAM-1 induction. Indeed, Murakami et al. [25] demonstrated that microvascular glomerular endothelial cells express less PECAM-1 than do human umbilical vein endothelial cells (HUVEC) and respond to TNF stimulation with decrease of PECAM-1 expression. The ability of an endothelial cell specific adhesion molecule to regulate proliferation might be crucial in characteristic endothelial behavior requiring mitogenesis, namely wound healing under physiological conditions and tumor angiogenesis when the system is dysregulated. Conversely, it is attractive to speculate that treatment of tumor vasculature with anti-PECAM-1 modalities might lead to inhibition of vessel growth and therefore tumor regression.

The mechanisms mediating β-catenin induction in PECAM-1 expressing cells remain speculative. The results of our present study conclude that PECAM-1 mediated β-catenin induction is not solely due to physical binding of the two proteins, implicating an indirect pathway for our observation. Moreover, the ability of the Δ11–16 PECAM-1 construct to increase transcriptionally active β-catenin, as judged by luciferase assays, suggests that the ectodomain of PECAM-1 with a small portion of the cytoplasmic region is at least, in part, responsible for the increase in β-catenin expression. The mechanism underlying this observation could be twofold. The ectodomain of PECAM-1, known to affect adhesion [26] may relay signals promoting accumulation of β-catenin or the cytoplasmic domain of PECAM-1 encoded by exon 10 may via the Wnt signaling pathway stabilize cellular β-catenin.

GSK3-β [31], casein kinase-1 (CK-1) [28,34], and casein kinase-II [4] play critical roles in stabilization of

β-catenin in the Wnt signaling pathway. CK-1, a serine–threonine kinase, phosphorylates Dvl-1, APC, axin, and β-catenin in vitro, leading to a dissociation of the β-catenin degradation complex [10], causing reduced GSK3-β-mediated phosphorylation and degradation of β-catenin. Given the role of PECAM-1 as a scaffolding molecule for various signaling proteins, CK-1 might bind to PECAM-1 in order to mediate subsequent phosphorylation events and thus increase β-catenin levels. These and other possibilities are being considered in our laboratory to elucidate the pathway mediating PECAM-1 induced β-catenin expression.

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