Mechanisms of VE-cadherin Processing and Degradation in Microvascular Endothelial Cells*

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VE-cadherin is an endothelial-specific cadherin that plays important roles in vascular morphogenesis and growth control. To investigate the mechanisms by which endothelial cells regulate cadherin cell surface levels, a VE-cadherin mutant containing the non-adhesive interleukin-2 (IL-2) receptor extracellular domain and the VE-cadherin cytoplasmic tail (IL-2R-VE-cadcyto) was expressed in microvascular endothelial cells. Expression of the IL-2R-VE-cadcyto mutant resulted in the internalization of endogenous VE-cadherin and in a dramatic decrease in endogenous VE-cadherin levels. The internalized VE-cadherin co-localized with early endosomes, and the lysosomal inhibitor chloroquine dramatically inhibited the down-regulation of VE-cadherin in cells expressing the IL-2R-VE-cadcyto mutant. Chloroquine treatment also resulted in the accumulation of a VE-cadherin fragment lacking the β-catenin binding domain of the VE-cadherin cytoplasmic tail. The formation of the VE-cadherin fragment could be prevented by treating endothelial cells with proteasome inhibitors. Furthermore, inhibition of the proteasome prevented VE-cadherin internalization and inhibited the disruption of endothelial intercellular junctions by the IL-2R-VE-cadcyto mutant. These results provide new insights into the mechanisms of VE-cadherin processing and degradation in microvascular endothelial cells.

Endothelial adherens junctions are adhesive intercellular contacts that are crucial for the maintenance and regulation of normal microvascular function (1–5). Alterations in adherens junction assembly influence endothelial cell motility, vascular morphogenesis, and permeability. Moreover, recent studies indicate that components of adherens junctions also function in intracellular signaling, leading to the current view that these complexes are plasma membrane domains that integrate chemical and mechanical signaling information (4). The major cell-cell adhesion molecule at endothelial adherens junctions is VE-cadherin, a cadherin family member that is specifically expressed in endothelial cells (5). The cytoplasmic tail of the classic cadherins, including VE-cadherin, comprises two well-characterized domains. The juxtamembrane domain (JMD) binds to the catenin p120, an armadillo family protein that is thought to regulate cadherin adhesive interactions by modulating the activity of Rho family GTPases (6–8). At the carboxy-terminal region of the cadherin cytoplasmic tail, a domain termed the catenin binding domain (CBD) interacts with β-catenin or plakoglobin (9). β-Catenin and plakoglobin both interact with α-catenin, which links cadherins to the actin cytoskeleton and to other actin-binding proteins such as α-actinin (10–13). VE-cadherin also associates with the vimentin cytoskeletal network in endothelial cells through interactions with plakoglobin and the intermediate filament-binding protein desmoplakin (14). These unique intercellular junctions, containing both actin and vimentin-binding proteins, have been referred to as complexus adhaerentes (15–17).

Although our understanding of the mechanisms of junction assembly has advanced significantly, much less is known about how cellular levels of cadherins are regulated. The loss of cadherin expression in epithelial cells is associated with a variety of pathologies, including tumor metastasis (18), and changes in cadherin expression are associated with epithelial-mesenchymal transitions during development (19). Interestingly, initiation of endothelial-mesenchymal transdifferentiation was found to correlate with the disruption of cell-cell contacts and has been marked by a loss of VE-cadherin expression in endothelial monolayers (20). A recent study (21) found that down-regulation of VE-cadherin in intimal neovessels was closely related to intimal inflammation. These data suggest that regulated changes in VE-cadherin levels have important consequences on endothelial function and pathophysiology. However, the cellular mechanisms that control cadherin expression and presentation at the cell surface are poorly characterized. Some insights into these issues have come from studies in which dominant negative mutants of cadherins were expressed in epithelial cells (22, 23). In a variety of model systems, cadherin mutants with a non-adhesive extracellular domain disrupt cell-cell adhesion (24) and decrease cell proliferation (25). The mechanism by which these mutants disrupt adhesion is not fully understood; however, several studies (26–28) have indicated that dominant negative cadherin mutants cause the down-regulation of endogenous cadherins. Using a mutant VE-cadherin in which the extracellular domain of VE-cadherin was replaced with the non-adhesive IL-2 receptor, we...
Recent studies indicate that some pools of cadherin on the cell surface are endocytosed and recycled back to the plasma membrane (30, 31). These observations suggest that cadherin endocytosis is an important regulatory mechanism that controls cadherin cell surface levels and perhaps regulates overall levels of cadherin expression. Because these endogenous cadherins are down-regulated by mutant cadherins, we hypothesized that these mutants trigger the endocytosis and degradation of endogenous cadherins. This model system might therefore be used to reveal the cellular machinery involved in regulating cadherin levels in vascular endothelial cells. In the present study, we utilized an adenoviral system to introduce the dominant negative IL-2R-VE-cadcyto mutant into primary cultures of microvascular endothelial cells (MECs). The results of this study indicate that expression of the IL-2R-VE-cadcyto mutant caused a rapid and dramatic down-regulation of endogenous VE-cadherin. Furthermore, the mutant triggered the internalization of endogenous VE-cadherin, which was subsequently processed through the endosomal-lysosomal pathway. Lysoosomal inhibitors prevented VE-cadherin degradation and revealed the formation of a VE-cadherin fragment that is generated during endocytic processing. The internalization and fragmentation of VE-cadherin could be blocked using proteasome inhibitors, suggesting a role for the proteasome in cadherin processing. Together, these observations provide new insights into the mechanisms by which cell surface levels of cadherins are regulated in microvascular endothelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of dermal microvascular endothelial cells (MECs) from human neonatal foreskin were purchased from the Emory Skin Diseases Research Center (Core B) and cultured in MCDB131 medium (Invitrogen, Carlsbad, CA). The culture medium was supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1-glutamine (Hernnd, VA), cAMP (Sigma, St. Louis, MO), hydrocortisone (Sigma), epidermal growth factor (Intergen, Purchase, NY), and antibiotic/antimycotic (Invitrogen). Cells were typically cultured 24–48 h and grown to 80% confluence for most experiments. For some experiments, MECs were seeded onto Matrigel to induce migration and grown to 80% confluency for most experiments. For some experiments, MECs were seeded onto Matrigel to induce migration and grown to 80% confluency for most experiments.

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adenovirus encoding the empty vector or the IL-2R-VE-cad cyto mutant (Fig. 2). An antibody directed against the VE-cadherin extracellular domain (Cad-5), the myc epitope tag, and vimentin. Note that the VE-cadherin antibody is directed against the VE-cadherin extracellular domain and thus recognizes only endogenous VE-cadherin. The IL-2R-VE-cad cyto mutant and all of the mutant VE-cadherin polypeptides expressed in MECs (see Fig. 2B) were routinely observed as a doublet by Western blot analysis. The polypeptides are thought to be differentially processed forms of the IL-2 receptor extracellular domain, because identical results were obtained using antibodies directed against either the VE-cadherin cytoplasmic tail or with antibodies directed against the IL-2 receptor (see also Panel C). As a control, an IL2-R-α5 cyto chimeric integrin was also expressed in MECs. No changes in VE-cadherin levels were detected in MECs expressing the IL2-R-α5 cyto chimeric integrin at modest (1 μl) or high (3 μl) titers of adenovirus relative to empty virus controls (C).

**Fig. 1.** The IL-2R-VE-Cad cyto mutant down-regulates endogenous VE-cadherin. A and B, primary human microvascular endothelial cells (MECs) were infected with empty virus or the IL-2R-VE-Cad cyto mutant for various amounts of time. Western blot analysis was carried out using antibodies directed against the VE-cadherin extracellular domain (Cad-5), the myc epitope tag, and vimentin. Note that the VE-cadherin antibody is directed against the VE-cadherin extracellular domain and thus recognizes only endogenous VE-cadherin. The IL-2R-VE-cad cyto mutant and all of the mutant VE-cadherin polypeptides expressed in MECs (see Fig. 2B) were routinely observed as a doublet by Western blot analysis. The polypeptides are thought to be differentially processed forms of the IL-2 receptor extracellular domain, because identical results were obtained using antibodies directed against either the VE-cadherin cytoplasmic tail or with antibodies directed against the IL-2 receptor (see also Panel C). As a control, an IL2-R-α5 cyto chimeric integrin was also expressed in MECs. No changes in VE-cadherin levels were detected in MECs expressing the IL2-R-α5 cyto chimeric integrin at modest (1 μl) or high (3 μl) titers of adenovirus relative to empty virus controls (C).

**Fig. 2.** The IL-2R-VE-Cad cyto mutant disrupts MECs intercellular junctions. MECs were infected with empty adenovirus (A and D), the IL-2R-VE-Cad cyto mutant (B and E), or the IL2-R-α5 cyto chimeric integrin mutant (C and F) for 18 h. The cells were then fixed in methanol and processed for dual label immunofluorescence using antibodies directed against endogenous VE-cadherin (A–C), the myc epitope tag (D and E), or the IL-2 receptor extracellular domain (F). Bar, 50 μm.

denovirus encoding the empty vector or the IL-2R-VE-cad cyto mutant (Fig. 2). An antibody directed against the VE-cadherin extracellular domain (cad-5) was used to specifically identify endogenous VE-cadherin and not the mutant, which lacks the VE-cadherin extracellular domain. The mutant cadherin was detected using antibodies directed against the myc epitope tag.
present at the carboxyl terminus. In uninfected MECs (not shown) and in control cells expressing empty adenoviral vector, extensive VE-cadherin staining was observed at MEC cell borders (Fig. 2A). In striking contrast, in MEC cultures expressing the IL-2R-VE-cadcyto mutant, endogenous VE-cadherin was distributed in a punctate cytoplasmic distribution (Fig. 2B). This punctate distribution was observed in virtually every cell expressing the IL-2R-VE-cadcyto mutant. Expression of the IL-2R-VE-cadcyto mutant had no effect on PECAM-1 protein localization (Fig. 3, A and C). Bar, 50 μm. E. MECs were infected with empty virus or the IL-2R-VE-Cadcyto mutant for 10 h. Western blot analysis was carried out using antibodies directed against VE-cadherin, PECAM-1, the myc epitope tag, and vimentin.

**Fig. 3.** Expression of the IL-2R-VE-Cadcyto mutant disrupts PECAM-1 localization but does not down-regulate PECAM-1 levels. MECs were infected by empty adenoviral vector (A and B) or the IL-2R-VE-Cadcyto (C and D) mutant for 10 h. The cells were then fixed in paraformaldehyde plus 0.5% Triton X-100 and processed for dual-label immunofluorescence using antibodies directed against PECAM-1 (B and D) and the myc epitope tag (A and C). Bar, 50 μm. E. MECs were infected with empty virus or the IL-2R-VE-Cadcyto mutant for 10 h. Western blot analysis was carried out using antibodies directed against VE-cadherin, PECAM-1, the myc epitope tag, and vimentin.

**VE-cadherin Is Internalized and Processed through the Endosomal/Lysosomal Pathway**—To determine if the punctate staining for VE-cadherin in cells expressing the IL-2R-VE-cadcyto mutant represented an internalized pool of protein, MECs were fixed in paraformaldehyde to detect cell surface VE-cadherin (Fig. 4A) or paraformaldehyde followed by Triton X-100 extraction to detect internalized VE-cadherin (Fig. 4B). No punctate staining for VE-cadherin was observed in cells fixed in paraformaldehyde, but a loss of cell surface cadherin staining was observed in cells infected with adenovirus expressing the IL-2R-VE-cadcyto mutant (Fig. 4A, asterisk). In contrast, in cells fixed in paraformaldehyde followed by permeabilization with Triton X-100, obvious VE-cadherin punctate staining was observed, indicating that the punctate VE-cadherin staining represents an internalized pool rather than cell surface clusters (Fig. 4B). These results indicate that dominant negative cadherin mutants induce internalization of endogenous VE-cadherin in primary human microvascular endothelial cells.

To determine if internalized VE-cadherin is destined for the endosome-lysosome pathway, co-localization of endogenous VE-cadherin with EEA-1, a marker for early endosomes, was carried out using dual-label immunofluorescence analysis (Fig. 4, C–E). As shown in Fig. 4E, the endogenous VE-cadherin localized to cytoplasmic vesicles containing early endosome antigen-1 (EEA-1), indicating that the internalized VE-cadherin was processed through the endosomal pathway. To determine if the internalized VE-cadherin was degraded via a lysosomal pathway, uninfected MECs and MECs expressing IL-2R-VE-Cadcyto were treated with the lysosomal inhibitor chloroquine. Interestingly, a dramatic increase in vesicular VE-cadherin staining was observed in uninfected MECs after treatment with chloroquine for 8 h (Fig. 5B), indicating that lysosomal inhibitors increase vesicular VE-cadherin accumulation even in quiescent MECs. In MECs expressing the IL-2R-VE-Cadcyto mutant, ~90% of the cells expressing this mutant exhibited vesicular VE-cadherin staining even in the absence of chloroquine (Fig. 5C). Treatment of MECs expressing the mutant cadherin with chloroquine resulted in a slight increase in vesicular cadherin, and the vesicular structures were often larger in chloroquine treated cells (Fig. 5D). Together, these results suggest that, in quiescent MECs, VE-cadherin is normally internalized and degraded via a lysosomal pathway and that the expression of cadherin mutants dramatically accelerates this process.

**Lysosome Inhibitors Prevent VE-cadherin Degradation and Reveal the Formation of a VE-cadherin Fragment**—The above results demonstrate that endogenous VE-cadherin is internalized and that the expression level of endogenous VE-cadherin is decreased upon the introduction of dominant negative cadherin mutants. Furthermore, the internalized VE-cadherin is processed through the endosome-lysosome pathway. To deter-
mine if lysosomal inhibitors could prevent the decrease in VE-cadherin levels in cells expressing the mutant cadherin. MECs that were infected with empty virus or IL-2R-VE-cad<sub>cyto</sub> were treated with chloroquine, and Western blot analysis was performed to evaluate the expression level of endogenous VE-cadherin. As shown in Fig. 6A, chloroquine treatment prevented the down-regulation of VE-cadherin in MECs expressing the IL-2R-VE-cad<sub>cyto</sub> mutant. The regulation of VE-cadherin expression was explored further by seeding MECs onto Matrigel, which induces rapid endothelial cell migration and the formation of branching networks (29, 36). As shown in Fig. 6B, VE-cadherin levels were dramatically decreased in MECs seeded onto Matrigel for 6 h. To determine if the down-regulation of VE-cadherin in endothelial cells induced to migrate on Matrigel could be inhibited with chloroquine, MECs were allowed to adhere to Matrigel or plastic for 1 h, and then...
Western blot using cad-5 antibody. In PBS, and then transferred to normal medium without chloroquine for various amount of time. The VE-cadherin fragment was monitored by Western blot using the cad-5 antibody. Alternatively, MECs were treated with chloroquine for 6 h, washed extensively, MECs seeded onto tissue culture plastic were treated with chloroquine for various amounts of time, and the appearance of the VE-cadherin fragment was monitored by Western blot using the cad-5 antibody. Alternatively, MECs were treated with chloroquine for 6 h, washed extensively in PBS, and then transferred to normal medium without chloroquine for various amount of time. The VE-cadherin fragment was monitored by Western blot using cad-5 antibody. D, quantification of VE-cadherin fragment levels at each time point. Each panel shown is representative of at least three independently conducted experiments.

treated with chloroquine for 6 h. Similar to MECs expressing the IL-2R-VE-cad_CytO mutant, chloroquine treatment attenuated the down-regulation of VE-cadherin in MECs seeded onto Matrigel. These data suggest that lysosomal degradation of VE-cadherin is used to control VE-cadherin expression in migrating endothelial cells.

Surprisingly, treatment of MECs with chloroquine also resulted in the appearance of a 95-kDa VE-cadherin fragment in both control MECs, and in MECs expressing the mutant cadherin (Fig. 6A). The amount of the VE-cadherin fragment generated in chloroquine-treated cells was also increased in MECs seeded onto Matrigel compared with control cells seeded onto plastic (Fig. 6B). This VE-cadherin fragment likely represents an intermediary in the processing of VE-cadherin during endocytosis and degradation. To determine the kinetics of the appearance of the VE-cadherin fragment in chloroquine-treated cells, time course experiments were conducted on uninfected MECs. MECs were treated with chloroquine for 0–9 h, and the appearance of the fragment was monitored by Western blot using an antibody directed against the VE-cadherin extracellular domain (ECD Ab). As shown in Fig. 6C, a steady increase in the accumulation of the VE-cadherin fragment is observed over this time frame. To determine how rapidly the fragmented VE-cadherin is subsequently processed for complete degradation, MECs were treated with chloroquine for 6 h and then transferred to normal growth medium without chloroquine for various amount of time. Three hours after the return to normal medium, the fragment was almost completely absent, indicating that the chloroquine treatment is reversible (Fig. 6D). The results of these experiments suggest that chloroquine treatment halts VE-cadherin degradation in the lysosome subsequent to an earlier cleavage of the VE-cadherin tail. Removal of chloroquine then allows the process to continue, resulting in complete lysosomal degradation of the partially processed VE-cadherin.

Internalized VE-cadherin Is Cleaved at a Site Very Close to the Boundary between the JMD and CBD—To determine the site at which the endogenous VE-cadherin is cleaved, VE-cadherin constructs encoding full-length VE-cadherin, a ΔCBD-VE-cadherin, or a VE-cadherin construct lacking the entire cytoplasmic domain (Fig. 7A) were used to evaluate the size of the VE-cadherin fragment observed in chloroquine-treated MEC cultures. The constructs were transiently transfected into COS cells, and Western blot analysis was performed in parallel with lysates from MECs treated with chloroquine. The cad-5 antibody recognizes an epitope within amino acids 91–105 (37) of the extracellular domain of human VE-cadherin and, therefore, detects all of the VE-cadherin deletion constructs as well as the fragment present in MEC lysates. As shown in Fig. 7B, the VE-cadherin fragment generated in MECs treated with chloroquine co-migrated with the ΔCBD-VE-cadherin deletion mutant, which lacks the β-catenin binding domain. This result suggests that the fragment observed in MECs treated with chloroquine represents a VE-cadherin polypeptide that was cleaved at a site very close to the boundary between the p120 and β-catenin binding domains. We further confirmed that the cleavage removed the catenin binding domain of VE-cadherin by using a second antibody directed against the carboxyl-terminal domain of VE-cadherin (ICD Ab). As shown in Fig. 7C, the extracellular domain antibody (ECD Ab) recognizes both the intact and truncated VE-cadherin, whereas the intracellular domain (ICD Ab) antibody only recognizes the intact VE-cadherin. These data confirm that the VE-cadherin fragment...
represents a truncation product lacking the β-catenin binding domain of the VE-cadherin tail.

To determine if the VE-cadherin tail is cleaved at the plasma membrane or after internalization, a trypsinization protocol was developed to distinguish the internalized pool of VE-cadherin from the cell surface pool. Trypsinization was used to remove cell surface proteins but leave intracellular protein pools intact (Fig. 8). In non-trypsinized cells, the IL-2R-VE-cadcyto mutant caused the down-regulation of endogenous VE-cadherin (Fig. 8A). When untreated cells were trypsinized before lysis in SDS-PAGE sample buffer, no VE-cadherin could be detected using the Cad-5 antibody directed against the VE-cadherin extracellular domain (Fig. 8B). These data demonstrate that the trypsinization protocol eliminated all cell surface VE-cadherin. Similar to the results shown above, chloroquine treatment prevented the down-regulation of VE-cadherin in MECs expressing the IL-2R-VE-cadcyto mutant (Fig. 8C). Furthermore, chloroquine treatment resulted in the accumulation of an intracellular pool of intact VE-cadherin that was trypsin-resistant (Fig. 8D). In MECs expressing the IL-2R-VE-cadcyto mutant, the majority of internalized VE-cadherin was intact, indicating that cleavage of the VE-cadherin tail is not required for internalization. These results demonstrate that the disruption of endothelial intercellular junctions is coupled to VE-cadherin internalization and suggest that the cleavage of the VE-cadherin tail occurs after the cadherin is internalized.

Proteasome Activity Is Required for VE-cadherin Internalization and Fragmentation—Recent studies have identified a role for the ubiquitin-proteasome system in the endocytosis of certain transmembrane proteins (38). To determine if the proteasome is involved in VE-cadherin endocytosis, MECs expressing the IL-2R-VE-cadcyto mutant were treated with chloroquine, the proteasome inhibitor MG132, or both chloroquine and MG132. Western blot and immunofluorescence experiments were performed to determine the expression level and localization of endogenous VE-cadherin in cells treated with proteasome inhibitors. Similar to chloroquine, MG132 prevented the down-regulation of VE-cadherin caused by dominant negative
VE-cadherin mutants (Fig. 8E). Furthermore, VE-cadherin internalization and the formation of the VE-cadherin fragment in chloroquine-treated cells were completely prevented by MG132 (Fig. 8, F and H). Similar results were obtained using the proteasome inhibitor lactacystin (not shown). These results indicate that VE-cadherin internalization and cleavage require an active proteasome system.

The above results suggest that cadherin mutants disrupt intercellular junctions by triggering endocytosis of endogenous cadherins. Because MG132 prevented VE-cadherin internalization, proteasome inhibitors were tested for the ability to block the dominant negative effects of VE-cadherin mutants in MECs. Immunofluorescence analysis was performed using an antibody directed against endogenous VE-cadherin in MECs expressing the IL-2R-VE-cadcyto mutant in the presence or absence of chloroquine and MG132. As shown in Fig. 9, intercellular junctions were disrupted in cells expressing the IL-2R-VE-cadcyto mutant, both without treatment (Fig. 9A) and with chloroquine treatment (Fig. 9B). However, MG132 (Fig. 9C) and lactacystin (not shown) dramatically inhibited the disruption of intercellular junctions by the IL-2R-VE-cadcyto mutant and prevented the appearance of intracellular vesicular VE-cadherin. These results are consistent with the trypsinization experiments shown in Fig. 8 and indicate that inhibition of the proteasome prevents VE-cadherin internalization. Western blot experiments confirmed that MG132 did not alter expression levels of the VE-cadherin mutants (not shown). These results demonstrate that an active proteasome system is required for the disruption of intercellular junctions by cadherin dominant negative mutants.

**DISCUSSION**

The results of the present study indicate that the expression of a VE-cadherin mutant in primary human microvascular endothelial cells induced extensive internalization and degradation of endogenous VE-cadherin. Importantly, VE-cadherin internalization and degradation was also observed in control cells, indicating that VE-cadherin endocytosis is part of a normal regulatory mechanism that endothelial cells utilize to control the adhesive properties of the plasma membrane. The internalized VE-cadherin was processed through the endosome-lysosome pathway but may also require an active proteasome system for internalization and degradation. Interestingly, cleavage of the VE-cadherin cytoplasmic tail at a site very close to the boundary between the p120 and β-catenin binding domains occurred during endocytic processing. These findings reveal new insights into the mechanisms by which cell surface levels of cadherins are regulated in microvascular endothelial cells.

In most previous studies, stable cell lines expressing the cadherin mutants were generated. In these models, analysis of junction assembly and endogenous cadherin levels is carried out after the mutant and endogenous cadherins reach steady-state levels. In the present study, we utilized an adenoviral delivery system. By rapidly inducing expression of the mutant cadherin with the adenoviral system, we were able to investigate early changes in VE-cadherin localization after the introduction of the mutant. Previous studies have shown that dominant negative cadherin mutants cause down-regulation of cadherins and increase cadherin turnover (27, 28), but the mechanisms driving these changes in cadherin levels were previously unknown. Interestingly, in addition to altering VE-cadherin levels in MECs, expression of the IL-2R-VE-cadcyto mutant in A431 cells also caused the down-regulation of endogenous E-cadherin (not shown). These data indicate that the effects of cadherin mutants are similar in different cell types, raising the possibility that the mechanisms that regulate VE-cadherin levels in MECs are similar to those regulating E-cadherin expression in epithelial cells. In MECs, expression of the mutant cadherin resulted in the appearance of a vesicular, cytoplasmic pool of the endogenous cadherin. The down-regulation of VE-cadherin was prevented by agents that inhibit lysosomal proteases, indicating a central role for the endosome-lysosomal pathway in regulating cadherin expression. The effects on VE-cadherin down-regulation were specific in that no changes in PECAM-1 levels were observed in MECs expressing the IL-2R-VE-cadcyto mutant (Fig. 3E). However, PECAM-1 accumulation at intercellular junctions was disrupted in MECs expressing the mutant cadherin (Fig. 3), indicating a role for VE-cadherin in regulating PECAM-1 distribution.

The mechanism by which VE-cadherin is internalized by dominant negative cadherin mutants is unclear. It is likely that competition between the exogenous mutants and the endogenous cadherins for cadherin-binding proteins such as p120 and β-catenin play some role during the endocytosis of endog-
enous VE-cadherin. Recently, the juxtamembrane domain of E-cadherin was found to interact with a cbl-related protein termed Hakai, an E3 ligase that participates in the ubiquitination of the E-cadherin cytoplasmic domain (39). Hakai interacts with E-cadherin, which is phosphorylated in a region near the p120 binding domain, and appears to target E-cadherin for internalization. However, VE-cadherin lacks two tyrosine residues that are required for Hakai binding to E-cadherin, and the membrane proximal sequence of the VE-cadherin cytoplasmic tail is highly divergent from other classic cadherins. Nonetheless, as part of a related analysis of VE-cadherin regulation by our laboratory, the p120 binding domain of the IL-2R-VE-cad<sub>het</sub> mutant was found to be necessary and sufficient to induce the internalization of endogenous VE-cadherin. These data suggest that binding partners for the cadherin juxtamembrane domain play an important role in regulating VE-cadherin turnover. It will be of interest to determine if Hakai-related proteins are expressed in endothelial cells and regulate VE-cadherin endocytosis, or if VE-cadherin internalization is regulated by mechanisms distinct from those described for E-cadherin.

Previous studies in epithelial cells indicated that the β-catenin binding domain on the mutant cadherin was required for the down-regulation of endogenous E-cadherin (25, 27). Several observations suggest an important role for the β-catenin binding domain in cadherin trafficking. Previous studies have demonstrated a role for β-catenin in targeting E-cadherin to the plasma membrane after synthesis, supporting a role for β-catenin in regulating cadherin metabolic stability (40). In the absence of β-catenin, the E-cadherin cytoplasmic domain is unstructured and exhibits increased susceptibility to proteolysis. The β-catenin binding domain of the E-cadherin cytoplasmic tail was recently reported to contain sequences that resemble a PEST motif, which is important in protein stability (41). In the MEC model system presented here, the catenin binding domain of VE-cadherin was removed after VE-cadherin internalization, and this process could be prevented by agents that inhibit the proteasome. The VE-cadherin cytoplasmic tail exhibits several regions of similarity to E-cadherin within the catenin binding domain. It is tempting to speculate that the disruption of the cadherin-catenin complex exposes sequences within the cadherin tail that target the cadherin for internalization and cleavage by the proteasome or by other cytoplasmic proteases.

Treatment of MECs with chloroquine resulted in the accumulation of a VE-cadherin fragment that was generated in both control MECs as well as MECs expressing the VE-cadherin mutants. This finding indicates that the processing of VE-cadherin represents a normal aspect of VE-cadherin metabolism, which is accelerated by the expression of the mutant cadherins. These observations raise the possibility that endocytosis of VE-cadherin might be accelerated during angiogenesis or in response to agents that disrupt endothelial barrier function. MECs seeded onto Matrigel exhibited a dramatic decrease in VE-cadherin levels compared with MECs seeded in parallel onto plastic substrates (Fig. 6). The down-regulation of VE-cadherin in MECs seeded onto Matrigel was inhibited by chloroquine treatment, which also resulted in the accumulation of the same VE-cadherin fragment observed in MECs expressing the mutant cadherin. These data suggest that the rates of VE-cadherin internalization are modulated by various stimuli that regulate cell endothelial cell migration or vascular barrier function. Consistent with this possibility, VE-cadherin internalization has been associated with alterations in endothelial monolayer permeability in vitro (42, 43).

During endocytosis, VE-cadherin is cleaved within the cytoplasmic tail near the boundary of the JMD and CBD. This determination is based on the fact that cadherin mutants lacking the CBD co-migrate with the fragment generated in MECs treated with chloroquine (Fig. 7). Furthermore, antibodies directed against the extracellular domain of VE-cadherin recognize the fragmented cadherin, but antibodies directed against the cadherin-terminal tail of VE-cadherin do not detect the truncated protein. The cleavage also appears to occur after the cadherin is internalized. In MECs expressing the IL-2R-VE-cad<sub>het</sub> mutant, an internal pool of full-length VE-cadherin is observed after trypsin treatment, indicating that the cadherin can be internalized without being cleaved (Fig. 8D). A number of recent reports indicate that cadherin cytoplasmic tails are substrates for several cellular proteases, including γ-secretase (44) and caspases (45, 46). We were unable to block VE-cadherin fragmentation during endocytosis with either caspase inhibitors or calpain inhibitors (not shown). In contrast, proteasome inhibitors MG132 (Fig. 8) and lactacystin (not shown) blocked both the internalization and fragmentation of VE-cadherin. It is formally possible that VE-cadherin endocytosis requires ubiquitination of the cadherin tail and that the use of proteasome inhibitors depletes cellular ubiquitin pools, thereby preventing VE-cadherin endocytosis (47). However, increasing evidence indicates that the proteasome pathway is involved in the regulation of lysosomal degradation of transmembrane receptors (48–50). In the case of VE-cadherin, the proteasome was also required for internalization of VE-cadherin. Furthermore, the disruption of intercellular junctions by dominant negative cadherin mutants could be prevented by proteasome inhibitors (Fig. 9). These results strongly suggest that cadherin mutants disrupt intercellular junctions by inducing endocytosis of the endogenous cadherins and that cadherin endocytosis might be utilized by endothelial cells to modulate endothelial barrier function during inflammation and angiogenesis.

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