T1α/podoplanin is essential for capillary morphogenesis in lymphatic endothelial cells

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Navarro A, Perez RE, Rezaiekhaligh M, Mabry SM, Ekekezie II. T1α/podoplanin is essential for capillary morphogenesis in lymphatic endothelial cells. Am J Physiol Lung Cell Mol Physiol 295: L543–L551, 2008. First published July 25, 2008; doi:10.1152/ajplung.90262.2008 — The lymphatic vasculature functions to maintain tissue perfusion homeostasis. Defects in its formation or disruption of the vessels result in lymphedema, the effective treatment of which is hampered by limited understanding of factors regulating lymph vessel formation. Mice lacking T1α/podoplanin, a lymphatic endothelial cell transmembrane protein, have malformed lymphatic vasculature with lymphedema at birth, but the molecular mechanism for this phenotype is unknown. Here, we show, using primary human lung microvascular lymphatic endothelial cells (HMVEC-LLy), that small interfering RNA-mediated silence of podoplanin gene expression has the dramatic effect of blocking capillary tube formation in Matrigel. In addition, localization of phosphorylated ezrin/radixin/moesin proteins to plasma membrane extensions, an early event in the capillary morphogenic program in lymphatic endothelial cells, is impaired. We find that cells with decreased podoplanin expression fail to properly activate the small GTPase RhoA early (by 30 min) after plating on Matrigel, and Rac1 shows a delay in its activation. Further indication that podoplanin action is linked to RhoA activation is that use of a cell-permeable inhibitor of Rho inhibited lymphatic endothelial capillary tube formation in the same manner as did podoplanin gene silencing, which was not mimicked by treatment with a Rac1 inhibitor. These data clearly demonstrate that early activation of RhoA in the lymphangiogenic process, which is required for the successful establishment of the capillary network, is dependent on podoplanin expression. To our knowledge, this is the first time that a mechanism has been suggested to explain the role of podoplanin in lymphangiogenesis.

lymphedema; lymphatics; GTPases; RhoA; Rac1; Matrigel

LYMPHATIC VESSELS PARTICIPATE in tissue fluid homeostasis and provide an important immunological defense for the body by enabling the secondary peripheral lymphoid tissues to offer the right microenvironment for the proliferation and expansion of immune cells specific to foreign antigens. Defining with clarity the factors controlling the lymphangiogenic process will benefit treatment of diseases such as cancer that rely on establishment of lymphatic vascularization to propagate to distant sites. Moreover, people suffering from lymphedema may benefit.

The major lymphangiogenic factors known in mammals are VEGF-C and VEGF-D, which act through their cognate receptors, Flt-4 (VEGFR-3). This receptor, which in the embryo is present in all endothelial cells, later becomes restricted in expression to only those of the lymphatic vasculature by term gestation (15). Numerous strategies to increase lymphatic vascular function (and prevent or ameliorate lymphedema) using VEGF-C have been tested in different animal models with some encouraging results (17, 21, 42). However, despite these and other significant progress in the field of lymphangiogenesis in the past few years, much remains to be uncovered about the factors involved and their mechanisms of action. The accumulation of new knowledge from identification and characterization of molecular markers that allow specific identification of lymphatic endothelial cells, like Prospero-related homeobox-1 (PROX1), lymphatic endothelial hyaluronan receptor-1 (LYVE-1), VEGF receptor-3 (VEGFR-3), and T1α/podoplanin offers new prospects for better understanding of lymphangiogenesis (1, 30). T1α/podoplanin is a type 1 transmembrane protein expressed by a number of cell types, but in endothelial cells, it is expressed only in those of lymphatic origin. The functional role of T1α/podoplanin is yet to be fully uncovered. It is referred to by many different names, like T1α or lung alveolar type I cell protein RTI40 (11, 33, 40), E11 antigen in rat osteoblasts and osteocytes (37), PA2.26 in mouse epidermal keratinocytes (10, 35, 36), product of mouse Ots-8 gene (29), murine gp38, canine gp40, human gp36 (9, 43, 44), and Aggrus or platelet aggregation-inducing factor for its ability to cause platelet aggregation when overexpressed on the surface of some tumor cells (16, 18, 19). The name podoplanin was assigned to describe its role in determining the shape of foot processes in rat kidney podocytes (4, 24). Recent papers suggest a potential role of podoplanin in epithelial-mesenchymal transition, in tumor invasion, and in the metastasis of tumors to the pulmonary microvasculature (20, 22, 23, 38, 39).

Generation of a knockout mouse model of T1α/podoplanin revealed its critical role in lung development, with homozygous animals being viable but dying shortly after birth due to respiratory failure, and with defects in alveolarization and anomalous proliferation of mesenchyme and epithelium in the lung likely due to a failure to generate type I epithelial cells (32, 34). Microarray analysis of the lungs at different stages of development have recently uncovered downregulation of the key cell cycle regulatory protein p21Waf1/Cip1 and a parallel increase in proliferating cell nuclear antigen as responsible for the hyperproliferation of peripheral lung cells in full-term gestation knockout mice (27).

A striking feature of the podoplanin homozygous knockout mouse phenotype is the presence of defects in lymphatic vessel pattern formation (with no alteration in blood vessels) that results in impaired lymphatic transport, dilation of lymphatic vessels, and congenital lymphedema (34). It was our objective to specifically study the role of podoplanin in lymphatic cap-
illary tube formation in vitro by using human primary lung lymphatic endothelial cells and small interfering RNA (siRNA) technology and question if podoplanin deficiency compromises capillary tube formation in lymphatic cells. Since the integrity of the tubular network is dependent on an intact cytoskeleton, and the Rho family of small GTPases mediate cytoskeletal dynamics (13), our attention was driven to the analysis of small GTPase activation in cells with reduced podoplanin expression. Also, because PA2.26 (mouse equivalent to human podoplanin) has been shown to physically interact with the actin-binding proteins ezrin and moesin in mouse carcinoma cell lines (35), we were prompted to analyze if the expression, phosphorylation, or cellular distribution of these molecules are affected when podoplanin levels are reduced.

**EXPERIMENTAL PROCEDURES**

**Antibodies and reagents.** Rat monoclonal against human podoplanin (clone NZ-1) was obtained from AngioBio (Del Mar, CA); goat polyclonal against actin was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA); rabbit polyclonal against human Prox1 was obtained from Upstate (Millipore, Billerica, MA); rabbit polyclonal against phosphorylated ERK phospho-erzrin (Thr567/radixin (Thr564)/moesin (Thr558) and ERK ezrin/radixin/moesin were obtained from Cell Signaling Technology (Danvers, MA); rabbit monoclonal against RhoA and rabbit monoclonal against Cdc42 were from Cell Signaling Technology (Danvers, MA); mouse monoclonal against Rac1 was from Cytoskeleton (Denver, CO); goat serum was obtained from Lonza (Walkersville, MD); fish skin gelatin was obtained from Sigma-Aldrich (St. Louis, MO); paraformaldehyde was obtained from Electron Microscopy Sciences (Hatfield, PA); PBS was obtained from Mediatech (Herndon, VA); Matrigel was obtained from BD Biosciences (Bedford, MA). All other reagents, unless indicated, were obtained from Sigma-Aldrich.

**Cell culture.** Primary human lung lymphatic microvascular endothelial cells (HMVEC-L) and blood lung microvascular endothelial cells (HBMEC-L) were obtained from Lonza and grown in EGM2-MV media, which consists of EBM-2 basal media plus 5% FBS and EGM2-MV growth media. Cells were observed under an inverted microscope Olympus IX50 at regular intervals, and pictures were taken after 2 and 16 and/or 18 h.

**Western blot analysis.** Cell lysates were prepared as described in Navarro et al. (28) with minor modifications. Lysis buffer consisted of 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, with complete miniprotease inhibitor tablet obtained from Roche Diagnostics (Indianapolis, IN), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerol phosphate, and 1 mM vanadate. After rinsing the plates with ice-cold PBS, 0.5 ml of lysis buffer was added to the plates, and the cells were scraped and transferred to eppendorf tubes and allowed to incubate on ice for 20 min with periodontexterning. After 20 min, samples were spun for 15 min at 12,000 rpm, and supernatants were removed and saved. Protein was determined using Bio-Rad DC protein assay, and equal protein amount samples were prepared and mixed with SDS × Laemml buffer, heated at 95°C for 10 min, and then run on SDS-PAGE gels (PAGE) Duramanide obtained from Lonza). After transferring the protein to PVDF membranes (Immobilon-P, Millipore), membranes were blocked with Casein Blocker in PBS from Pierce for 30 min, then briefly rinsed with PBS-Tween, and incubated overnight with the primary antibodies, always ranging 1/500 to 1/1,000, made in a solution of 1% chicken egg ovalbumin (from Sigma-Aldrich). After rinsing with PBS-Tween and incubating with appropriate secondary antibodies (HRP-conjugated ECL; GE-Healthcare Amersham, Pittsburgh, PA) and four more rinses with PBS-Tween, chemiluminescent substrate ECL was added to the membranes, and signal was detected using a conventional X-ray film-developing automated system.

**Immunofluorescent staining.** Cells plated onto p100 dishes were control siRNA or podoplanin siRNA transfected and incubated for 48 h; they were then seeded onto p100 dishes (8–9 × 105 cells/dish) that had been coated with 9 ml of Matrigel that was immediately aspirated. Plates were returned to the incubator for the indicated times, and, after washing with ice-cold PBS, pH 7.4, cells were treated with 8 ml of BD Cell Recovery Solution obtained from BD Biosciences (San Jose, CA) at 4°C for 2 h. After complete release from the gel, cells were centrifuged at 500 g for 5 min at 4°C, washed once with ice-cold PBS, and then lysed in 0.4 ml of ice-cold lysis buffer (50 mM Tris·HCl, pH 7.5, 10 mM MgCl2, 500 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail). After lysis for 20 min on ice, cell debris was removed by centrifugation at 3000 g for 10 min at 4°C, protein was determined for the different samples, and half of each lysate (100 μg protein) was mixed with 15 μg of rhodamin-RBD Protein Agarose beads (50 μg of protein, as recommended by the manufacturer, Cytoskeleton) or 20 μl of PAK-GST beads (20 μg) and incubated for 1 h at 4°C with rotation. Samples were then centrifuged (5,000 rpm for 1 min at 4°C) and washed twice in ice-cold wash buffer (25 mM Tris·HCl, pH 7.5, 30 mM MgCl2, and 40 mM NaCl), finally resuspended in 30 μl SDS × Laemml buffer and heated at 100°C for 5 min, and then separated on 12% polyacrylamide gels and processed for Western blot after transferring to Immobilon membranes.

**Immunofluorescent staining.** Sterile round glass cover slips were placed onto a 24-well plate and coated with 180 μl/well of Matrigel that was immediately aspirated to form a thin coat on the
Podoplanin silencing in human lung lymphatic microvascular endothelial cells has no effect on cell viability or cell morphology in tissue culture. Podoplanin homozygous knockout mice are born with lymphedema caused by capillary leak from lymphatic vessels (and die of respiratory failure within minutes of being born due to a failure to properly inflate the lungs), presenting enlarged intestinal and cutaneous lymphatic vessels. We decided to address the role of podoplanin in lymphatic vasculature by using an in vitro cell culture system of primary human lung microvascular lymphatic endothelial cells (HMVEC-LLy) by means of siRNA. Because primary cultures of microvascular endothelial cells are classically hard to transfet, we first tested transfection conditions of these primary cells using Lipofectin and a fluorescent oligonucleotide (Block-iT-FITC, both reagents from Invitrogen) achieving, surprisingly, 85–90% transfection efficiency as addressed right after transfection (Fig. 1A). We then used an siRNA specific for human podoplanin (siRNA podo) and were able to decrease the expression of the protein as determined by Western blot 48 h after transfection with 100 nM siRNA (Fig. 1B), compared with a non-targeting siRNA control with exact nucleotide composition (designed using the online tool from Invitrogen). Cells transfected with siRNA podoplanin remained viable in tissue culture (>96% after 3 and 4 days), and they were morphologically indistinguishable from siRNA control-treated cells 3 days after transfection (Fig. 1, C and D); the cell population was devoid of visible dead cells, although reducing podoplanin expression appeared to slow cell proliferation in culture (data not shown).

**RESULTS**

Podoplanin silencing in human lung lymphatic microvascular endothelial cells has no effect on cell viability or cell morphology in tissue culture. Podoplanin homozygous knockout mice are born with lymphedema caused by capillary leak from lymphatic vessels (and die of respiratory failure within minutes of being born due to a failure to properly inflate the lungs), presenting enlarged intestinal and cutaneous lymphatic vessels. We decided to address the role of podoplanin in lymphatic vasculature by using an in vitro cell culture system of primary human lung microvascular lymphatic endothelial cells (HMVEC-LLy) by means of siRNA. Because primary cultures of microvascular endothelial cells are classically hard to transfet, we first tested transfection conditions of these primary cells using Lipofectin and a fluorescent oligonucleotide (Block-iT-FITC, both reagents from Invitrogen) achieving, surprisingly, 85–90% transfection efficiency as addressed right after transfection (Fig. 1A). We then used an siRNA specific for human podoplanin (siRNA podo) and were able to decrease the expression of the protein as determined by Western blot 48 h after transfection with 100 nM siRNA (Fig. 1B), compared with a non-targeting siRNA control with exact nucleotide composition (designed using the online tool from Invitrogen). Cells transfected with siRNA podoplanin remained viable in tissue culture (>96% after 3 and 4 days), and they were morphologically indistinguishable from siRNA control-treated cells 3 days after transfection (Fig. 1, C and D); the cell population was devoid of visible dead cells, although reducing podoplanin expression appeared to slow cell proliferation in culture (data not shown).

Formation of capillary networks in Matrigel by human lung lymphatic microvascular endothelial cells requires podoplanin expression. Inspired by the in vivo phenotype of T10/podoplanin−/− mice, we performed tube formation assays in Matrigel using primary human lymphatic endothelial cells transfected with control or podoplanin siRNA to test the hypothesis that podoplanin regulates lymphatic endothelial cell function when cells are exposed to a substrate matrix that resembles more closely the in vivo environment. As shown in Fig. 2, A and B, decreasing podoplanin expression has a dramatic effect on tube formation by HMVEC-LLy in Matrigel, with tube-like structures being disrupted, shorter, and simplified with respect to control siRNA-transfected cells (the number of tubes per field is reduced to 40 ± 12% that of control, and the number of intersections in the network or “knots” is reduced to 30 ± 15%). To determine the specificity of this effect, we performed the same experiment using human lung blood microvascular endothelial cells (HBMEC-L) that do not express podoplanin (due to its blood-derived origin instead of lymphatic). As shown in Fig. 2, C and D, transfec-
tion of HBMVEC-L with podoplanin siRNA, as expected, had no effect in capillary tube formation (number of tubes per field is 85 ± 12% that of control siRNA cells, and the number of knots is kept at 82 ± 15%). Transfection efficiency was higher than 80% (not shown). Endothelial cells derived from blood vessels do not express podoplanin and can form capillary tubes by alternative means, and only lymphatic endothelial cells require podoplanin for the complete formation of capillary tubes.

RhoA activation in Matrigel is impaired in lymphatic endothelial cells with decreased podoplanin expression. Members of the family of small Rho GTPases like Rho, Rac, and Cdc42 are known modulators of signal transduction pathways that regulate cytoskeletal dynamics (12, 13), and RhoA and Rac1 activities are required for the initial steps of morphogenesis in capillary network formation (6). To determine what possible signaling pathway is modulated by podoplanin, we addressed the changes in the activity of these GTPases in the absence of podoplanin, right after plating onto Matrigel. As shown in Fig. 3, RhoA-GTP levels rise quickly and transiently after plating onto Matrigel, being maximal by 30 min, as addressed by a pulldown assay using rhotekin-RBD bound to Sepharose beads; this activation is greatly diminished in podoplanin siRNA-transfected cells. A similar approach to reveal changes in Rac1 or Cdc42 activation by pulldown assays with the binding domain of p21-activated kinase (PAK) bound to Sepharose beads showed comparable activation of Rac1 between control siRNA- and podoplanin siRNA-transfected cells with a slight delay in the activation kinetics and no difference in Cdc42 activation. Remarkably, the basal level of active Rac1 and Cdc42 was much higher than that of RhoA in these cells.

Fig. 2. Podoplanin expression is required for capillary tube formation in HMVEC-LLy. Tube formation assay was performed as described in EXPERIMENTAL PROCEDURES, and photos shown represent cells after 18 h on Matrigel, as indicated × 20. A: HMVEC-LLy with control siRNA. B: HMVEC-LLy with podoplanin siRNA. C: HBMVEC-L with control siRNA. D: HBMVEC-L with podoplanin siRNA. Shown is a representative experiment out of at least 3 times.

Fig. 3. Time course of small GTPase activation during capillary tube formation in control siRNA- or podoplanin siRNA-transfected cells. Time courses obtained with pulldown assay as described in EXPERIMENTAL PROCEDURES are shown for RhoA (A), Rac1 (B), and Cdc42 (C). Total levels of GTPase in cell lysates, as well as podoplanin and actin expression, are shown for all experiments. The experiment was repeated 3 times and shown is a representative experiment.
RhoA activation in Matrigel, and not Rac1, is required for capillary tube formation in HMVEC-LLy. To test the possibility that the compromised activation of RhoA could explain the effect of decreased expression of podoplanin in capillary tube formation by these cells, we tested the effect of a novel cell-permeable inhibitor of Rho, CT04, which is highly purified C3 exotransferase covalently linked to a proprietary cell penetrating moiety via a disulfide bond (from CytoSkel). The cell-penetrating moiety allows rapid and efficient transport through the plasma membrane, and a pretreatment of just 2 h with 1 μg/ml of the inhibitor in serum-free media was able to prevent tube formation in HMVEC-LLy cells (Fig. 4, A and B), which was not prevented by the serum starvation alone. Cell viability was not affected by the treatment with the inhibitor, as tested by trypan blue exclusion after the treatment and just before plating onto Matrigel (viability >97%) and by cytotoxicity assays (WST-1 from Roche, data not shown). Treatment with the inhibitor was effective at reducing levels of RhoA-GTP as shown in Fig. 4C. Thus, podoplanin is required for normal RhoA activation in lymphatic endothelial cells undergoing capillary tube formation, and the lack of activation of the small GTPase could explain the failure in tubulogenesis.

Because we observed a modest effect on Rac activation by podoplanin knockdown, we decided to also address the possible role of Rac1 activation on capillary tube formation. As seen in Fig. 5, A and B, pretreatment of HMVEC-LLy with the specific Rac1 inhibitor NSC23766 (30 μM for 18 h) did not have any effect on the process of tube formation, but caused a significant inhibition on Rac1 activity as shown in Fig. 5C (decreased the level of active Rac1 as addressed by pulldown assay with PAK-PBD beads over 90%).

Proper cellular localization of phosphorylated ERM proteins during capillary morphogenesis requires podoplanin expression. ERM proteins have been implicated in the organization of specialized plasma membrane domains and in the control of cell shape, cell adhesion, and migration by linking transmembrane proteins to the cytoskeleton (5). Microvilli formation correlates with the localization of ERM family of actin-binding proteins to plasma membranes, and phosphorylation of ERM proteins in their COOH-terminal domain after growth factor treatment in fibroblastic cells such as A431 or NIH/3T3 causes their localization to what appears as microvilli at the plasma membrane (12, 26); its dephosphorylation causes translocation to the cytoplasm. PA2.26, the mouse equivalent to human podoplanin, has been shown to physically interact with ezrin and moesin in mouse carcinoma cell lines in culture (35). In some human oral squamous cell carcinomas, podoplanin has been shown to associate to ezrin in plasma membrane protrusions (22, 23), and in human MCF-7 cells overexpressing exogenous podoplanin, the actin cytoskeleton is reorganized (38, 39). Those observations prompted us to analyze the levels and cellular distribution of ERM proteins and their phosphorylated forms during the process of capillary morphogenesis by Western blot and immunofluorescence staining. Immunofluorescence staining in control siRNA- or podoplanin siRNA-transfected cells showed no differences in either levels or cellular distribution of ERM proteins while in culture, 48 h posttransfection (Fig. 6). A time course Western blot analysis for ERM and P-ERM in these cells, after inducing capillary tube formation by plating onto Matrigel, showed that there were no differences between these two types of cells (Fig. 7A). However, immunofluorescence staining of the same cells showed a clear perturbation in the distribution of P-ERM proteins to plasma membrane extensions, resembling microvilli, in cells with decreased podoplanin expression (Fig. 7, B and C).

DISCUSSION

Disruption of lymphatic vessels compromises lymph flow in vivo, and one of the major challenges in fighting the ensuing lymphedema becomes limited understanding of factors contributing to the formation and integrity of lymphatic vessels. On the other hand, diseases such as cancer rely on establishment of lymphatic vascularization through the process of lymphangiogenesis to spread to distant sites. In this respect, T1α/podoplanin, a lymphatic endothelial cell transmembrane protein, is of great interest, as it has been shown that mice lacking this protein have malformed and leaky lymphatic vasculature, but the molecular mechanism that explains this phenotype is un-

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**Fig. 4.** Rho inhibitor blocks tube formation in HMVEC-LLy. Cells were left untreated or treated with 1 μg/ml Rho inhibitor for 2 h before plating onto Matrigel. Photos taken at 2 (A) and 16 (B) h. Experiment was repeated at least 3 times, and photos shown correspond to a representative experiment (∗/40). C: to test if the inhibitor was effective, RhoA-GTP levels were determined in control or inhibitor-treated cells by pull-down assay as described in EXPERIMENTAL PROCEDURES.
known. We report here the first data showing that podoplanin expression in human lymphatic endothelial cells is necessary for lymphatic capillary tubulogenesis as assessed in Matrigel, and, importantly, describe the possible mechanism. The significance of our work is that it extends current understanding of podoplanin’s role in lymphatic endothelial cell function, specifically on lymphangiogenesis, by providing hitherto lacking mechanistic insight. Thus, it may be possible to inhibit lymphangiogenesis by targeted inhibition of podoplanin function.

To date, the only published work exploring the possible role of podoplanin in endothelial cell function is that of Schacht et al. (34) where it was reported that overexpressing a rat podoplanin construct in a hemangioendothelioma-derived mouse cell line (EOMA) or an immortalized human dermal microvascular endothelial cell line (HMEC-1) increased tube formation in Matrigel and cell migration/adhesion to collagen/fibronectin. Unfortunately, although the effect of decreasing podoplanin expression in human primary dermal lymphatic endothelial cells was also assessed in that study, it was restricted to analyzing only the effect on adhesion to collagen with no data on ability of the cells to form capillary tubes; this missing data limits the conclusion that could be drawn from the overexpression studies alone in cells of non-lymphatic origin, especially given the heterogeneity known to exist between endothelial cells of different origin as reflected in differentially expressed proteins other than podoplanin that may also affect their function (14, 31). Moreover, our own results point to existence of endothelial cell type-specific mechanisms for capillary tube generation, since blood vessel endothelial cells do not express podoplanin and yet form capillary tubes when

Fig. 6. Podoplanin expression does not alter levels of ezrin/radixin/moesin (ERM) proteins in HMVEC-LLy cells. A: lysates of control siRNA- or podoplanin siRNA-transfected cells were prepared 48 h after transfection, and Western blot was performed on 20 μg of protein; control siRNA- (B) or podoplanin siRNA- (C) transfected cells were stained for ERM (in red). Nuclei are shown in blue (DAPI; ×400).

Fig. 5. Rac1 inhibitor does not affect tube formation in HMVEC-LLy. Cells were left untreated or were treated with 50 μM Rac1 inhibitor for 18 h before plating onto Matrigel. Photos taken at 2 (A) and 16 (B) h; the experiment was repeated 3 times and shown is a representative set of results (×40). C: to test if the inhibitor was effective, Rac1-GTP levels were determined in control or inhibitor-treated cells by pulldown assay as described in Experimental Procedures.
plated on Matrigel. Podoplanin is required only in cells of lymphatic nature; thus, blood vascular endothelial cells must use an alternative pathway.

In vivo, new blood vessel formation from preexisting vasculature (angiogenesis) or de novo formation from progenitors/precursors (vasculogenesis) requires proliferation of endothelial cells, branching, sprouting, and lumen formation to establish tubular networks. This morphological differentiation has been well studied for endothelial cells of blood vascular origin, but less so for those of lymphatic origin. The process is exquisitely dependent on interaction of endothelial cells with the extracellular matrix, proper cell-cell contacts and communication, protease activities, and the presence of cytokines and growth factors. Recreating the process of capillary tube formation in vitro in Matrigel as in our experiments does not require cell proliferation and occurs within hours, providing a simple and reproducible method to study the angiogenic process. Although results cannot be directly extrapolated to in vivo angiogenesis, this model is of great help in dissecting the mechanisms involved in the process and is often corroborated later by findings in in vivo models of angiogenesis.

Lumen formation and branching during the formation of new capillaries requires the formation and coalescence of intracellular vacuoles with combined action of actin filaments and microtubules, being completely dependent on the cytoskeletal machinery. Rho GTPases are required for the formation of vacuoles and lumen, in particular Rac1 and Cdc42 (2). The microtubular cytoskeleton is essential in maintaining and stabilizing the endothelial network, with microtubule-depolymerizing agents inducing endothelial network collapse and apoptosis dependent on RhoA (3). The activities of Rac1 and RhoA seem to be required for the initial steps of morphogenesis in capillary network formation, including the formation of adherens junctions and the remodeling of the vascular network and mechanical stretching of cells (6, 7). In the study described here, the lymphangiogenic process in primary human lymphatic endothelial cells is accompanied by an early increase in RhoA activation and a slower activation of Rac1. The basal activation level is very low for RhoA, and this activation appears completely dependent on the expression of podoplanin. Activation of Rac1 seems to be delayed in the absence of podoplanin, but RhoA inhibitor treatment, and not Rac1 inhibitor, causes the same effect as podoplanin knockdown in these cells. In conclusion, our data clearly demonstrates that early activation of RhoA in the lymphangiogenic process, which is required for the successful establishment of the capillary network, is dependent on podoplanin expression. To our knowledge, this is the first time that a mechanism has been suggested to explain the role of podoplanin in lymphangiogenesis.

ERM actin-binding proteins have been implicated in the organization of specialized plasma membrane domains and in the control of cell shape, cell adhesion, and migration by linking transmembrane proteins to the actin cytoskeleton (5). When inactive, their COOH-terminal domain is associated through intramolecular interactions with their NH2-terminal domain (FERM domain, for band 4.1, ezrin, radixin, moesin homology domain). This FERM domain can associate with integral membrane proteins. In the active state, both domains are exposed, and the protein is allowed to interact with membrane proteins while the COOH terminus binds actin filaments, and these activated ERM proteins and their binding partners are involved in the organization of microvilli. Specific residues in the COOH-terminal domain of ERM proteins can be phosphorylated by the RhoA-dependent kinase ROCK in vitro (25), and the association of ERM with plasma membranes is dependent on Rho activity (not Rac1 or Cdc42) but independent of ROCK (Rho-kinase). Microvilli formation correlates with the localization of ERM to plasma membranes, and phosphorylation of ERM proteins in their COOH-terminal domain after growth factor treatment in fibroblastic cells like A431, or NIH/3T3, causes their localization to what appears as microvilli in the plasma membrane (6, 26) with dephosphoryla-
tion causing translocation to the cytoplasm. This seems to be dependent on the cell type used because in epithelial cells like MDCK, LLC-PK1, and MDBK, ERM proteins can be activated in a Rho-independent manner, and they are stabilized in their active form independently of phosphorylation, apparently by a mechanism dependent on polyphosphoinositide generation. Interestingly, in some human oral squamous cell carcinomas, podoplanin has been shown to associate with ezrin in plasma membrane protrusions (22), and in human MCF-7 cells overexpressing exogenous podoplanin, it reorganizes the actin cytoskeleton (38, 39). Overexpression of podoplanin in dermal keratinocytes increases their motility and decreases cell-cell adhesions strength (36). In the same cells, podoplanin has an effect on cell-cell adhesiveness and is involved in reorganizing the actin cytoskeleton by recruitment of ezrin and induction of cell-surface protrusions. In our experiments, we find that podoplanin does not change the level of phosphorylated ERMs but is clearly involved in the proper recruitment of phosphorylated ERM proteins to plasma membrane protrusions resembling microvilli. We observe that in cells at a relatively high density in culture, the phosphorylation level of ERM proteins is low, independently of podoplanin levels; phosphorylation increases quickly after plating onto Matrigel, and, although the level of P-ERM is not changed, localization of these proteins to microvilli appears to be compromised in cells that have low podoplanin expression. We have not been able to see physical interaction between podoplanin and ERM proteins by standard biochemical techniques (immunoprecipitation, not shown) and are still pursuing the identification of potential podoplanin-binding proteins at the plasma membrane or in the extracellular matrix that could regulate its biological activity.

In summary, our findings indicate there is a critical requirement for podoplanin function in the lymphangiogenic process. This raises the prospect that targeted blockade of lymphangiogenesis via interference with podoplanin function could be an effective strategy to limit tumor lymphatic vascularization and hence metastatic spread.

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