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Vascular Endothelial Growth Factors C and D Induces Proliferation of Lymphangioleiomyomatosis Cells through Autocrine Crosstalk with Endothelium

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Lymphangioleiomyomatosis (LAM) is a potentially fatal lung disease characterized by nodules of proliferative smooth muscle-like cells. The exact nature of these LAM cells and their proliferative stimuli are poorly characterized. Herein we report the novel findings that the lymphangiogenic vascular endothelial growth factors (VEGF) C and D induce LAM cell proliferation through activation of their cognate receptor VEGF-R3 and activation of the signaling intermediates Akt/mTOR/S6. Furthermore, we identify expression of the proteoglycan NG2, a marker of immature smooth muscle cells, as a characteristic of LAM cells both *in vitro* and in human lung tissue. VEGF-C-induced LAM cell proliferation was in part a result of autocrine stimulation that resulted from cross talk with lymphatic endothelial cells. Ultimately, these findings identify the lymphangiogenic VEGF proteins as pathogenic growth factors in LAM disease and at the same time provide a novel pharmacotherapeutic target for a lung disease that to date has no known effective treatment. *(Am J Pathol 2009, 175:1410–1420; DOI: 10.2353/ajpath.2009.080830)*

The understanding of lymphatic biology has expanded rapidly with the discovery of the lymphangiogenic proteins vascular endothelial growth factors (VEGF) C and D and their cognate receptor VEGF-R3.[1,2] They are members of the VEGF family of growth factors, having 40% homology with VEGF-A, and their importance in lymph vasculature biology is well demonstrated by the finding that VEGF-C null mice die as a result of abnormal lymphatic development.[3] Increasingly it has become clear that the biological role of these VEGF proteins have expanded beyond effects on the lymphatic endothelium alone to include the peri-lymphatic milieu and the investing immature vascular smooth muscle or pericytes.[4] Furthermore, they have now been implicated in both pathogenic and non-pathogenic human processes including cancer growth and metastasis, wound healing, and immune regulation.

The lymphatic endothelial cell (LEC) is distinct from blood vessel endothelium with respect to their biological function, structure, and protein repertoire.[5] As such they represent a unique cell that may play a distinctive role in the pathogenesis of human disease. Like blood vessel endothelium they share their basement membrane with surrounding immature smooth muscle cells called pericytes. It is clear that along with VEGF, pericyte-endothelial cell interactions play an important role in modulating vascular biology.[4]

Until recently the lymphatic vasculature has been considered an innocent bystander in the pathogenesis of pulmonary disease. Historically, one such disease has been pulmonary lymphangioleiomyomatosis (LAM), a progressive and fatal lung disease that almost exclusively affects women in their reproductive years.[6,7] Pathologically, LAM is characterized by proliferation of abnormal smooth muscle-like cells that form lung nodules distributed in a peri-lymphatic manner. The etiology of LAM has been linked to mutations in the *TSC2* gene; however, the nature and site-of-origin of LAM cells remains speculative.[8] Based on the LAM cells proximity to lymphatic vessels, we hypothesized that the LEC and lymphangiogenic proteins VEGF-C and/or -D might play a role in their proliferation; and that these smooth muscle-

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like LAM cells represented perivascular mural cells that were proliferating through a mechanism involving LEC cross talk.

Using both *in vitro* primary LAM-derived cells (LDC)[9] and immunohistochemistry of human lung LAM tissues, we report that VEGF-C and -D induce LDC proliferation through activation of their cognate receptor VEGF-R3 and subsequently phosphatidylinositol 3-kinase (PI3K)/mTOR/S6 signaling. Of note, this VEGF-R3 proliferative signaling pathway did not appear to be inhibited by functional tuberin. Furthermore, LDC proliferation was induced by cross talk between LEC and LDC and mediated through LDC production of VEGF-C. Taken together, these results identify the lymphangiogenic VEGF proteins as novel pathogenic growth factors in LAM disease and the lymphatic endothelium as a modifying factor in LAM cell proliferation.

Materials and Methods

Chemicals and Reagents

Recombinant human VEGF-C, -D and mutated VEGF-C (Cys156Ser, a VEGF-R3 specific agonist), platelet-derived growth factor (PDGF), non-conjugated or biotin conjugated antibodies to VEGF-R1, VEGF-R2 or VEGF-R3, respectively, and the VEGF-C/D-binding chimeric protein VEGF-R3/Fc (human VEGF-R3 extracellular domain fused to the carboxy-terminal Fc region of human IgG1) were purchased from R&D Systems (Minneapolis, MN). The VEGF-R3 inhibitor, MAZ-51 was purchased from Alexis Biochemicals (San Diego, CA). Wortmannin, rapamycin, and antibodies to β-actin and α-smooth muscle actin were purchased from Sigma (St. Louis, MO). Monoclonal antibody to VEGF-R3 (M-20) was purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Antibodies to total and phospho-mTOR (ser 2448), Akt, phospho-Akt (ser473), S6-ribosomal protein, phospho-S6 (ser 235/236) (2F9), P70S6 Kinase, phospho-p70 S6 kinase (Thr389), ERK-2, phospho-ERK (Thr202/Tyr204) (D13.14), Tuberin TSC2, and Hamartin TSC1 were obtained from Cell Signaling Technologies (Danvers, MA). Antibodies to Prox-1, NG2, and phospho-tyrosine (4G10) were purchased from Millipore-Upstate (Lake Placid, NJ). Phycoerythrin and streptavidin conjugated goat anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Cells and Cell Culture

Primary LAM-derived smooth muscle-like cells (clone 5/50 and 12/89) [9] passages 3 to 10 were grown in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA), and constituents as previously described.[10] These cells have been well characterized and were derived from nodules of separate patients with LAM that express tuberin but have one allele of the *TSC2* gene that is mutated. All presented data are from either of the two LDC clones and are representative of the similar findings obtained by using either clone.

Primary adult human microvascular endothelial cells and pulmonary arterial smooth muscle cells (PASM) were obtained from Cambrex (East Rutherford, NJ) and grown in cell specific growth factor-supplemented nutrient media as per the manufacturer's instructions (Cambrex, EBM-2). The microvascular endothelial cells all expressed the LEC marker, Prox-1,[11] and thus served as a model of LEC for co-culture experiments.

For expression of *wt-TSC2*, LDC were seeded in six well plates at 2×10^5 per well and grown in full growth media until 70% to 80% confluent. Cells were then transfected with lentivirus carrying green fluorescent protein tagged *wt-TSC2* (human) or the GFP alone (kind gifts of V. Krymskaya), by using methods as previously described.[10] Forty-eight hours post-transfection, cells were serum starved for 24 to 48 hours before all experiments; growth factor stimulation at 200 ng/ml for time intervals as indicated, in the presence or absence of various inhibitors.

Immunoblotting and Immunoprecipitation

LAM cells (2×10^5) were grown in six well-plates until 70% confluent, serum starved for 24 hours, and lysates were obtained after stimulation with VEGF-C (200 ng/ml) in the absence or presence of various inhibitors (200 nmol/L Wortmannin, 200 nmol/L Rapamycin or 10 μmol/L of MAZ51) for different time intervals as indicated. Cells were rinsed twice with ice-cold PBS/sodium orthovanadate (Na3VO4, 10 mmol/L) and then lysed with radioimmunoprecipitation assay buffer (20 mmol/L Tris pH-7.4; 150 mmol/L NaCl; 5 mmol/L EDTA; 1% Triton-X 100; 0.5% sodium deoxycholate; 10% glycerol; 25 mmol/L NaF; 1 mmol/L phenylmethylsulfonyl fluoride; and 1 mmol/L Na3VO4) with phosphatase and protease inhibitors (Sigma). Lysate protein concentration was measured by using the Bio-rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as standard. Lysate proteins (10 to 15 μg) were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA). The membrane was blocked in 5% nonfat dry milk, and then probed with appropriate specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and signals were detected by using enhanced chemiluminescence method (Amersham, Piscataway, NJ).

For immunoprecipitation of VEGF-R3, LDC were stimulated with VEGF-C or VEGF-D (200 ng/ml) for 5 minutes and protein lysates were prepared by using the lysis buffer as described above. Pre-cleared lysates were incubated with 2 μg of anti-VEGF-R3 antibody bound to 30 μl of protein-A Sepharose beads (Amersham, Piscataway, NJ). The beads were washed, resuspended in Laemmli sample buffer, boiled at 95°C for 5 minutes, resolved on SDS-polyacrylamide gel electrophoresis under reducing conditions, proteins transferred to polyvinylidene difluoride membranes and immunoblotted with anti-phosphotyrosine antibody (4G10).