The role of vascular endothelial growth factor isoforms in lung development and neonatal lung disorders

Ph.D. Thesis

Dr. Galambos Csaba

School of Ph.D. Studies
Doctoral School of Pathology
Semmelweis University

Supervisor: Paku Sándor, Ph.D.

Budapest
2008

INTRODUCTION

The human lung is a complex, highly structured organ, in which a vast vascular network is intimately associated with an equally vast arbor of epithelial-lined tubes and sacs for the prime purpose of gas exchange. Lung vascular development is a carefully orchestrated multistep process regulated by a tight balance between angiogenic stimulators and inhibitors. This complex, highly organized, step series requires exquisite orchestration of the regulatory activity of multiple growth factors, matrix proteins, and cytokines in a specific temporo-spatial order. Most of these growth factors are members of three major growth factor families including Vascular Endothelial Growth Factor (VEGF), Angiopoietin and Ephrin families. Understanding the functional reach of several members of these growth factor families is integral to an appreciation of the etiology and pathogenesis of developmental lung vascular disorders affecting newborns and children.

One example, alveolar capillary dysplasia (ACD), a rare, fatal disease, is characterized by failure of formation of air-blood barriers, the presence of dysplastic, thin-walled vessels in the central part of the alveolar septa, and often by misalignment of pulmonary veins.

A likely candidate as a regulator for the formation of the lung's vasculature is vascular endothelial growth factor (VEGF). VEGF is so critical for embryonic development that in the mouse, elimination of just a single allele is lethal. In the early stages of lung development, the mouse VEGF gene expresses three isoforms (120, 164, and 188) via alternate mRNA splicing. The isoform have different physico-chemical characteristics and their expression shows a distinct temporo-spatial pattern and organ specificity. These features suggest that each isoform could serve specific function during lung vascular development and

PUBLICATIONS RELEVANT TO THESIS

1. Deutsch G, Young LR, Deterding R, Fan LL, Dell SD, Bean JA, Brody A, Langston C, and the Pathology Cooperative Group: Albright E, Askin F, Baker P, Chou P, Cool C, Coventry S, Cutz E, Davis MM, Dishop MK, **Galambos C**, Patterson K, Travis WD, Wert S, White F, M.D. Diffuse Lung Disease in Young Children: Application of a Novel Classification Scheme. *Am J Respir Crit Care Med*. **2007** Sep 20, in press.

2. **Galambos C,** deMello DE: Molecular Mechanisms in Pulmonary Vascular Development. *Pediatr Dev Pathol,* **2007;** 10:1-17.

3. **Galambos C.,** Nodit L.: Identification of lymphatic endothelium in pediatric vascular tumors and malformations. *Pediatr Dev Pathol*. **2005**; 8:181-9.

4. **Galambos C.,** Ng Y., Ali A., Noguchi A., Lovejoy S., D'Amore P., deMello D.E.: Defective pulmonary development in the absence of heparin binding VEGF isoforms. *Am J Respir Cell Mol Biol.* **2002**; 27:194-203.

6. D2-40 antibody reliably distinguishes lymphatic channels from vascular endothelium of misaligned veins; therefore it is a useful tool in the histologic diagnosis of ACD.

7. Altered VEGF isoform signaling may play a role in the pathogenesis of ACD.

8. VEGF120/120 mouse could be utilized as potential animal model of alveolar capillary dysplasia.

In summary, we propose that altered VEGF signaling is one of the causative factors in the pathogenesis of ACD and we speculate that the VEGF120 mouse could be further utilized to study the pathomechanisms of ACD.

that delicately balanced VEGF isoform concentration gradients are necessary for appropriate blood vessel morphogenesis.

We engineered mice that express only VEGF120 isoform, to study the role of VEGF isoforms in lung development**,** and to assess their possible involvement in the pathogenesis of ACD.

OBJECTIVES

1. To study the impact of the loss of VEGF isoforms 188 and 164 expression upon pulmonary vascular development, utilizing the VEGF120/120 transgenic mouse model.

2. To describe the morphologic changes of pulmonary vessels and airways of VEGF120/120 fetuses and pups by using a wide variety of techniques including light & transmission electron microscopy and immunohistochemistry of lung tissues, as well as scanning electron microscopy of lung vascular casts.

3. To compare the pulmonary phenotype of VEGF120/120 mice to that of alveolar capillary dysplasia (ACD).

4. To assess the ability of D2-40 antibody to identify lymphatic versus vascular endothelium in lung tissues obtained from patients with ACD.

5. To evaluate if VEGF120/120 mouse is suitable as animal model of ACD.

MATERIALS AND METHODS

Experimental animals

The generation of the VEGF120 isoform-specific mice was achieved by site-specific removal of VEGF gene exons 6 and 7 in embryonic stem (ES) cells using the Cre/LoxP system. Neonates expressing exclusively VEGF120/120, sired by VEGF+/120 breeding pairs, were recovered at birth. Correct gene targeting was confirmed by quantitative RNA analysis. To obtain VEGF120 isoform-specific embryos and newborn pups, VEGF120/+ heterozygous female mice were crossed with VEGF120/+ heterozygous male mice to obtain timed-pregnant female mice which was followed by embryo harvest.

Light and Transmission Electron Microscopy

Fetuses, aged E (gestation age) 9 and E10, were fixed by immersion in 2% glutaraldehyde. For fetuses and pups aged E15–PN (Postnatal day) 1, the lungs were removed from the thoracic cavity and immersed in 2% glutaraldehyde. Blocks were postfixed in osmium tetroxide (OsO_4). After dehydration in acetone, the blocks were embedded in Spurr (Polysciences Inc., Warrington, PA). Toluidine blue stained thin (1-µm) sections were screened for those containing predominantly peripheral lung tissue and not conducting airways, and these were sectioned for ultrastructural examination. Ultrathin sections examined with a JEOL 100 CX (JEOL USA, Inc., Peabody, MA) electron microscope.
For light microscopy, the specimens were fixed in 10% formaldehyde or 4% paraformaldehyde, dehydrated in graded alcohols and embedded in paraffin. 6-µm–thick sections were stained with hematoxylin-eosin, or collected on Super Frost plus

D2-40 staining in Alveolar Capillary Dysplasia

The D2-40 antibody specifically and reliably highlighted the lymphoid channels in the broncho-arterial units, and remained consistently negative in the endothelium of misaligned veins. The endothelial cells of misaligned veins of ACD, as well as of lymphoid channels stained positive with the panendothelial cell marker, CD31.

CONCLUSIONS

1. The VEGF120/120 mouse is an excellent developmental model of pulmonary vascular development.

2. The presence of VEGF164 and 188 isoforms is critical in mouse lung development.

3. VEGF 164 and/or 188 contribution to pulmonary pre-acinar vessel development is not significant.

4. In the mouse fetuses and pups, the absence of heparan-binding VEGF164 and 188 isoforms impairs the development of the lung's microvasculature and delays airspace maturation.

5. VEGF 164 and/or 188 has an exquisite dose dependent effect on air-blood barrier formation and pulmonary capillary development.

Sensitivity and Specificity of D2-40 Antibody for Lymphatic Endothelium

The lesions examined included 14 lymphatic malformations and 11 vascular lesions. Patients' ages ranged from 2 weeks to 16 years, and the locations of the lesions varied from the skin to internal organs. The clinical diagnosis by in large concurred with the pathology. In all cases D2-40 antibody labeled only the endothelium of thin-walled vascular channels, which was morphologically consistent with lymphatic vessels (25 of 25 cases). No staining of endothelial cells of non lymphatic vascular lesions or nonlesional arteries and veins (0 of 25) was observed. Five lymphatic lesions showed more than 75% D2-40– positive channels (category A), 5 lesions had approximately 50% (category B), and 4 lesions showed fewer than 25% D2-40–positive channels (category C). All lymphatic lesions had D2-40– positive vessels, but not all the vessels were positive. Some lymphatic vessels (mostly the large ones) stained only partly with D2-40. In general, the large, dilated lymphatic channels did not stain well with D2-40, although there were a few lesions with complete large vessel staining. In contrast, the small lymphatic vessels and the lymphoid tissue – associated small channels stained consistently well with D2-40. CD31 antibody consistently labeled the endothelium of arteries, veins, capillaries, and lymphatics (including small and large ones) in all lesions and endothelial cells in vascular lesions. The specificity of D2-40 antibody for lymphatic endothelial cells was virtually 100% (0% false positive), whereas sensitivity was estimated at 60% to 65% (35% to 40% false negative). CD31 antibody showed very high sensitivity for all endothelial cells (100%), but no specificity for lymphatic endothelium.

slides (Fisher Laboratories, Pittsburgh, PA) for immunohistochemical stains.

Immunostaining

Identification of Type I (T1) pneumocytes was performed using a monoclonal antibody (T1α) to T1 protein transcript. Immunohistochemistry was performed in combination with Tyramide Signal Amplification (Tyramide Signal Amplification kit , NEN Life Science Products Inc., Boston, MA).To determine peripheral vessel density within the lung, platelet-endothelial cell adhesion molecule (PECAM/CD31) staining of frozen sections of the lungs from E 18.5 fetuses of each of the three genotypes was done as follows: lungs were fixed in 4% paraformaldehyde overnight at 4°C and cryoprotected with 15% sucrose followed by 30% sucrose solution washes. The fixed lungs were then embedded in frozen tissue-embedding media (Fisher, Cat# SH75–1250). The sections were treated with 36% urea solution at 95 $\pm$ 50°C in a microwave oven for 10 min for antigen retrieval. The sections were incubated with the primary antibody against CD31 (PECAM-1, Cat# 550274; BD Pharmingen, San Diego, CA). After washing in PBS, sections were incubated in biotinylated anti-rat IgG (BD Pharmingen) at a 1:250 dilution and subsequently with 1:100 avidin-biotin–linked peroxidase (ABC Kit; Vector Laboratories, Burlingame, CA). The substrate used for detection was 0.1% 3.3'-diaminobenzidine, and Fast Green was used as a counterstain. Positive immunostaining for CD31 was revealed by brown staining of endothelial cells in the lumen of blood vessels.

Determination of Airspace–Parenchyma Ratios

The ratio of air spaces to parenchyma was determined by the Chalkley point-counting method. Toluidine blue-stained, 1-μm–thick sections of peripheral lung tissue from E15, and E16 fetuses and PN1 pups were digitized with a charge couple device (CCD) camera and the images displayed on a computer monitor. A transparency of a 315-point grid was superimposed on the monitor screen and the points overlying airspaces and lung parenchyma were counted respectively. The investigators were unaware of the genotype during this analysis. The airspace-parenchyma ratio was calculated and Student's t test was used to determine statistical significance between genotypes.

Quantitation of Air–Blood Barriers

Electron micrographs of 10 random fields of the lung from five PN1 pups of each genotype were used to determine the number of air-blood barriers per alveolus. During the analysis, the investigators were unaware of the genotype. An air–blood barrier was defined as an oval or round space, either empty or containing blood cells abutting the airspace lumen and bounded on one side by an endothelial cell. The Student's t test was used to determine statistical significance between genotypes.

Quantitation of Peripheral Vessels

PECAM/CD31 labeling was used as a marker of endothelial cells within the walls of peripheral lung vessels. Under the light microscope, the total number of PECAM-stained cells in 11 high-power fields of the lung sections from each genotype was counted. Five animals of age E15, E16, and PN1 were examined. The quantitation was conducted in a masked fashion and the investigators did not know the genotype during the analysis.

Vascular Casts, Scanning Electron Microscopy, and Generation Count of Pre-Acinar Vessels

The earliest gestational age at which a successful complete pulmonary vascular cast could be obtained for a VEGF120 homozygous fetus was E15. At all time points studied (E15, 17, 18, and P1), VEGF120 homozygous fetuses had casts that were smaller and less dense compared with the heterozygous and wild-type littermates. Analysis by SEM showed that the proximal vessel branches (pre-acinar vessels) were not significantly different in number or caliber (Figure 27, top), but the density of the peripheral vasculature was strikingly sparse in VEGF120 homozygous fetuses of all ages studied compared with heterozygous and wild-type littermates. In VEGF120 homozygous fetuses, the luminal diameter of these peripheral end-vessels was larger, and the morphologic profiles coarse compared with the heterozygous or wild-type littermates. In heterozygous fetuses, the overall size of the pulmonary vascular casts were not obviously different compared with the wild-type littermates, suggesting that growth of the larger vessels was not affected, but the smaller peripheral vasculature is decreased in density. These differences persisted through PN1, the latest age for which a pulmonary cast was examined. The size of the vascular casts reflects overall lung size. Throughout gestation, lung size was similar between wild-type and heterozygous littermates. However, the lungs of homozygous littermates were smaller by 0.5–1 mm in apex to base length compared with heterozygous and wild-type littermates. In addition, in E18.5 animals, the homozygous fetal lungs were relatively bloodless compared with wild-type and heterozygous littermates, reflecting the decreased peripheral vessel density seen in the casts. The size of the trachea and main bronchi however did not differ among the genotypes.

also, the airspace–parenchyma ratios of heterozygous E16 fetuses and PN1 pups were lower compared with their respective wild-type littermates. Transmission electron microscopy of lungs from E9 and E10 animals revealed capillaries containing hematopoietic cells in all three genotypes. At PN1, VEGF120 homozygous pups had fewer well-formed air blood barriers compared with wild-type littermates. Instead of the expected close apposition between the alveolar epithelium and the capillary endothelial cells seen in the alveoli of the wild-type mice, the most peripheral vessels in the VEGF120/120 mice were separated from the airspace lumen by 2–3 cell layers. The number of air–blood barriers as defined, per airspace was counted. VEGF120 homozygous animals had significantly fewer air–blood barriers, 1.15 per alveolus, compared with 4.65 in wild-type littermates ($P < 0.0001$). Heterozygous pups had an average of 3.45 air–blood barriers per alveolus, significantly greater than the VEGF120 homozygous littermates ($P < 0.001$) but still significantly less than wild-type littermates ($P < 0.009$).

Immunostaining for Type I Cells

There was no difference in the distribution of immunoreactivity for T1α protein within Type I cells in PN1 pups from the three genotypes.

Quantitation of Peripheral Vessel Density

In the homozygous E 18.5 fetus, peripheral lung vessel density was significantly reduced when compared with both the wild-type (384 versus 499/11 high-power fields [hpf]; $P < 0.03$), and the heterozygous littermates (488/11 hpf; $P < 0.03$). The vessel density in the heterozygous fetus did not differ from that in the wild-type littermate (488 versus 499/11 hpf; $P < 0.4$).

Vascular Casts and Scanning Electron Microscopy

To obtain lung vascular casts, the anterior chest wall was removed, and a freshly prepared mixture of Mercox catalyst and resin (Ladd Research Industries, Burlington, VT) at a 50:1 ratio was injected into the right cardiac ventricle through a 30-gauge needle, under a dissecting microscope. To clear soft tissue, the injected fetuses and pups were placed in 20% KOH for 7–10 d with daily changes of solution. Dried specimens were examined using a JEOL JSM-5800 scanning electron microscope (JEOL USA, Inc., Peabody, MA). Scanning electron microscopy was performed on lung vascular casts of fetuses aged E15–18, and PN1 pups.

Generation Counts of Pre-Acinar Vessels

In E15 fetuses, the number of generations of pre-acinar vessels was determined under scanning electron microscopy at low magnification (x43). At this early age, the development of the peripheral vasculature is not dense enough to obscure the proximal vessels, so that generation counts could be made. At later ages, the density of the peripheral vasculature obscures the pre-acinar vessels and precludes such study.

Specificity and Sensitivity of D2-40 Antibody to the Lymphatic Endothelium

Cases of 14 pediatric lymphatic and 11 vascular lesions were randomly selected and with D2-40 or CD31 antibodies. To identify lymphatic channels we used morphologic criteria (gold standards) that characterized lymphatic vessels as thin-walled channels lined with endothelial cells with or without valves, luminal proteinaceous material, and associated lymphoid tissue.

CD31 antibody (mouse monoclonal, clone JC70A) was purchased from Dako (Carpinteria, CA, USA) and D2-40 (mouse monoclonal, clone D2-40) from obtained from Signet (Dedham, MA, USA). Brown-colored staining of endothelial cells (cytoplasmic and membranous) was interpreted as positive. For specificity, D2-40 staining of arterial and venous endothelial cells of all lesions was assessed (non-endothelial cell types were not investigated). For sensitivity, lymphatic lesions were categorized based on the percentage of their D2-40–positive lymphatic channel content. Category A lesions were defined as having more than 75% of all vessels stained, category B lesions as approximately 50%, and category C lesions as fewer than 25% D2-40–positive lymphatic channels. Vessels with partial D2-40–positive endothelium were excluded. The percentage of D2-40–stained vessels was estimated independently by 2 investigators with approximately 90% agreement. In some instances, we found a few D2-40–positive channels in the surrounding tissue but clearly apart from the lesions. We interpreted these vessels (which were also positive for CD31) as a component of normal tissue. Because these channels generally were small and slit like, we could not apply our gold standards; hence, we were not able to identify them as lymphatic. Moreover, it was difficult to estimate the normal number and distribution of lymphatic channels adjacent to certain vascular lesions; therefore, these nonlesional vessels were not analyzed. In all lesions, the staining pattern of D2-40–stained vascular channels was compared with that of CD31-stained vessels. D2-40 and CD31 endothelial cell staining of vessels in the broncho-arterial units were evaluated in paraffin-embedded lung sections of patients with ACD.

RESULTS

Animal Genotype

Genotype of each fetus or pup was determined by PCR using genomic DNA. The PCR genotyping results were further confirmed by Southern blot analysis. The genotype distribution of the VEGF fetuses followed the normal Mendelian frequency with the vast majority being wild-type and heterozygous fetuses, while the least frequent fetus genotype was homozygous.

Determination of Airspace–Parenchyma Ratios and Air-Blood Barrier Count

In E15 and E16 fetuses, and PN1 pups, airspace maturation in VEGF120 homozygous mice was delayed. In VEGF120 E15 fetuses, there was a delay of at least one developmental stage of lung development compared with the wild-type littermate (pseudoglandular versus canalicular). To assess the degree of airway or airspace development, the ratio of airway/airspace to parenchyma was determined. In E15 VEGF120 homozygous animals, this ratio was significantly less than in wild-type littermates, 0.05 versus 0.1 ($P < 0.007$). In heterozygous animals, the airspaces were larger than in homozygous littermates, the airspace–parenchyma ratio being 0.0858 ($P < 0.08$). The difference between heterozygous and wild-type littermates did not reach statistical significance ($P < 0.19$). A reduction in the airspace–parenchyma ratios of VEGF 120 homozygous animals compared with wild-type animals was also present in E16 fetuses, 0.175 versus 0.322 ($P < 0.006$), and PN1 pups, 1.359 versus 2.478 ($P < 0.016$). In these older animals