Published in final edited form as:

Angiogenesis. 2018 November ; 21(4): 837–848. doi:10.1007/s10456-018-9628-3.

Heparin Impairs Angiogenic Signaling and Compensatory Lung Growth After Left Pneumonectomy

Duy T. Dao^{1,2}, Lorenzo Anez-Bustillos^{1,2}, Jared Ourieff^{1,2}, Amy Pan^{1,2}, Paul D. Mitchell³, Hiroko Kishikawa^{1,2}, Gillian L. Fell^{1,2}, Meredith A. Baker^{1,2}, Randolph S. Watnick^{1,2}, Hong Chen^{1,2}, Thomas E. Hamilton², Michael S. Rogers^{1,2}, Diane R. Bielenberg^{1,2}, and Mark Puder^{1,2,*}

¹Vascular Biology Program, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

²Department of Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

³Institutional Centers for Clinical and Translational Research, Boston Children's Hospital, Boston, MA 02115

Abstract

Children with hypoplastic lung diseases, such as congenital diaphragmatic hernia, can require life support via extracorporeal membrane oxygenation (ECMO) and systemic anticoagulation, usually in the form of heparin. The role of heparin in angiogenesis and organ growth is inconclusive, with conflicting data reported in the literature. This study aimed to investigate the effects of heparin on lung growth in a model of compensatory lung growth (CLG). Compared to the absence of heparin, treatment with heparin decreased the VEGF-mediated activation of VEGFR2 and mitogenic effect on human lung microvascular endothelial cells (HMVEC-L) *in vitro*. Compared to non-heparinized controls, heparinized mice demonstrated impaired pulmonary mechanics, decreased respiratory volumes and flows, and reduced activity levels after left pneumonectomy. They also had lower lung volume, pulmonary septal surface area and alveolar density on morphometric analyses. Lungs of heparinized mice displayed decreased phosphorylation of VEGFR2 compared to the control group, with consequential downstream reduction in markers of cellular proliferation and survival. The use of bivalirudin, an alternative anticoagulant that does not interact with VEGF, preserved lung growth and pulmonary mechanics. These results demonstrated that heparin impairs CLG by reducing VEGFR2 activation. These findings raise concern for the clinical use of heparin in the setting of organ growth or regeneration.

Keywords

vascular endothelial growth factor; compensatory lung growth; pneumonectomy; heparin; bivalirudin

*Correspondence: Mark Puder, MD PhD, Boston Children's Hospital, Department of Surgery, 300 Longwood Ave, Fegan 3, Boston, MA 02115, Phone: 617-355-1838, Mark.Puder@childrens.harvard.edu.

Introduction:

Since its discovery in the early 1900s and subsequent introduction into clinical applications in the 1930s-1940s, heparin has become one of the most widely used drugs in medicine[1] and currently remains a mainstay of anticoagulation therapy[2]. Beyond its well-known role as an anticoagulant, heparin and its closely related polysaccharide, heparan sulfate (HS), exert a wide range of effects in the fields of embryology, skeletal development, inflammation, wound healing, tumor metastasis, and angiogenesis[3–6]. The ability of heparin and HS to interact with major angiogenic growth factors is well established[7]. Different forms of exogenous heparin can act as antagonists by binding and sequestering fibroblast growth factor (FGF), thus disrupting its interaction with FGFR[8].

Vascular endothelial growth factor (VEGF), the master regulator of angiogenesis, is another growth factor that can interact with heparin due to its heparin binding domains (HBD)[9]. Analogous to its role in FGF signaling, exogenous heparin, especially at a lower molecular weight, inhibits VEGF's angiogenic effects on endothelial cells[10]. Multiple studies have discovered other heparin-like compounds that can exert similar inhibitory effects on angiogenesis in efforts to develop new anti-tumor therapies[11–15].

In addition to its well-studied roles in tumor growth and metastasis, VEGF also plays a critical role in other physiologic processes, such as organ regeneration[16]. Compensatory lung growth (CLG) after unilateral pneumonectomy is a unique process that is highly dependent on VEGF. Activation of VEGFR2 has been shown to be pivotal in CLG as mice with endothelial cell-specific *Vegfr2* knockout display impaired restoration of alveolar structure and function following unilateral pneumonectomy[17]. Based on the nature of the interactions between heparin and multiple angiogenic growth factors including VEGF, we hypothesized that systemic heparinization may significantly impact physiologic processes such as organ growth and regeneration. In this study, we seek to understand the interaction between heparin and VEGF during CLG after unilateral pneumonectomy and the physiologic impact of systemic heparinization on this process.

Methods:

VEGF Activation and Proliferation Assay:

For the activation assay, human microvascular lung endothelial cells (HMVEC-L) (Lonza, Morristown, NJ) were plated at 70% confluence on gelatin-coated and Matrigel®-coated plates (Corning, Corning, NY) in basal medium (EBM-2 medium (Lonza, Morristown, NJ) + 0.5% fetal bovine serum) and starved overnight at 37°C. Cells were washed after 12 hours and treated with basal medium, basal medium + 10 ng/mL VEGF₁₆₅, or basal medium + 10 ng/mL VEGF₁₆₅ + heparin ranging from 0–1 U/mL for 5 minutes at 37°C. All in vitro and in vivo experiments in this study were performed with unfractionated heparin (Hospira, Lake Forest, IL). The medium was then removed and cells were quickly lysed on ice with 1x Laemmli buffer (Boston Bio Products, Ashland, MA). The supernatant was then analyzed for P-Y1175-VEGFR2 level with immunoblot.

For proliferation assay, HMVEC-L were plated at 30% confluence on gelatin and Matrigel®-coated plates in basal medium and starved overnight at 37°C. Cells were washed after 12 hours and treated with basal medium, basal medium + 10 ng/mL VEGF₁₆₅, or basal medium + 10 ng/mL VEGF₁₆₅ + heparin ranging from 0–1 U/mL. After 3 days of incubation at 37°C, cell viability was assessed with Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) and colorimetric signal was detected with FLUOstar Omega microplate reader.

Assessment of Anticoagulation Functions:

Eight-week old C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME) received intraperitoneal (IP) injection of normal saline at 100 µL, heparin at 500 U/kg, or bivalirudin (Sigma Aldrich, St Louis, MO) at 50 mg/kg every 12 hours for 24 hours and a total of 3 doses. One hour after the third dose of injection, blood was collected via inferior vena cava venous puncture in 9:1 ratio with trisodium citrate 3.2%. Plasma was separated by centrifugation at 3000 g for 20 minutes. Analysis of heparin activity on the heparinized and saline-treated control mice was performed with a chromogenic anti-factor IIa activity assay (Aniara, West Chester, OH) according to the manufacturer's protocol. Coagulation functions, including prothrombin time (PT), partial thromboplastin time (PTT), and anti-factor Xa activity, of the control and bivalirudin-treated mice were measured by the core hematology laboratory at Boston Children's Hospital.

Effects of Systemic Heparin on Compensatory Lung Growth:

Surgical Model and Experimental Groups: All procedures were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital. Eight-week old C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME) were first anesthetized with ketamine 80–100 mg/kg and xylazine 10–12.5 mg/kg via intraperitoneal (IP) injection and underwent left pneumonectomy as previously described[18].

Mice were randomized into four experimental groups: control, heparin, VEGF, and VEGF + heparin. Mice in the control group received 100 µL of normal saline via IP injection immediately after surgery and daily thereafter. Mice in the heparin group were systemically heparinized as previously described[19]. Briefly, mice received IP heparin at 500 U/kg/dose immediately after surgery, followed by once every 12 hours for the first 48 hours after pneumonectomy and once every 24 hours for the next 48 hours[19]. The VEGF group received 0.5 mg/kg of recombinant murine VEGF₁₆₄ (GenScript, Piscataway, NJ) via IP injection. The heparin + VEGF group received both systemic heparinization and VEGF delivery in a similar fashion. Mice in all experimental groups were euthanized on post-operative day (POD) 4. This experimental time point was chosen based on previous work from our group, where lung growth was shown to be most active[20].

Plethysmography and Assessment of Activity Levels: On POD 4, mice underwent plethysmography studies (Emka Technologies, Falls Church, VA). Each mouse was placed in a sealed chamber, which measured flow and pressure changes that occur with respiration.

Mice were allowed 5 minutes of habituation, followed by 5 minutes of measurement of tidal volume, expiratory volume, minute ventilation (the product of tidal volume and respiratory rate), and inspiratory and expiratory flow. Following plethysmography studies, mice were then placed in a 42 × 42 cm open field where their traveled distance and basic and fine movements were recorded for 6 minutes with an infrared-based motion tracking system (Motormonitor, Kinderscientific, Poway, CA). Fine movements referred to instances when the animal broke the same beam of infrared without moving from its spot, e.g. grooming or head movements, while basic movements captured the number of times the animal blocked a new beam.

Lung Volume Measurement: Animals were weighed and euthanized with carbon dioxide on POD 4. The remaining right lung was removed en-bloc with the tracheobronchial tree. The lung was infused with formalin at 25 cmH₂O and total lung volume was measured with the water displacement method[21]. The trachea was then ligated and the specimen was placed in 10% formalin overnight at 4°C. All specimens were transferred into 70% ethanol after 24 hours of formalin fixation and subsequently embedded in paraffin for morphometric analyses. Lung volume was normalized for body weight.

Morphometric Analysis: Quantitative microscopy was performed on hematoxylin and eosin (H&E) stained lung sections based on the principles of stereology. Five lungs from each experimental group were represented by systematic uniform random sampling of 20–30 five-micron sections examined under light microscope at 200X magnification. Using a point and intersection counting technique, parenchymal volume, alveolar volume, and septal surface area were calculated[22, 23]. Parenchyma was defined as the gas-exchange compartment of the lung, which included alveoli, terminal ducts, and alveolar septa. All volume and area measurements were normalized for body weight. Alveolar density was mathematically calculated from counting the number of alveolar transections on 10 randomly chosen fields at 400X magnification using the method of Weibel[24].

Immunoblot and Quantitative Polymerase Chain Reactions (qPCR) Array of VEGFR2 Signaling: Fresh lung tissues from the control and heparin groups were suspended in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA). Tissue samples were then homogenized and centrifuged for 15 minutes at 4°C and 14,000 rpm. The supernatants were collected and 40 micrograms of proteins were used for immunoblot. Primary antibodies included anti-P-Y1175-VEGFR2, -VEGFR2, -P-T308-Akt, -P-S473-Akt, -Akt (Cell Signaling Technology, Danvers, MA), -VEGF 120/164 (R&D Systems, Minneapolis, MN) and -β-Actin (Sigma-Aldrich). Secondary antibodies included horseradish-peroxidase-conjugated anti-rabbit, anti-mouse (R&D Systems) and anti-rat IgG (Jackson ImmunoResearch, West Grove, PA).

Total RNA was extracted from lung tissues with the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. RNA concentration was determined with the NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed with the RT² First Strand Kit (Qiagen, Germantown, MD). Synthesized cDNA was then mixed with reaction reagents provided by

the RT² Profiler PCR Array for VEGFR2 (Qiagen, Germantown, MD). Amplification reactions were achieved with the StepOne Real-Time PCR Systems (Applied Biosystems, Foster City, CA). All target genes were normalized to the housekeeping gene β -Actin and their mRNA expression level was calculated using the 2^{-C_t} method[25].

Effects of Bivalirudin on Compensatory Lung Growth:

Experimental Groups: Mice were randomized into four experimental groups: control, bivalirudin, VEGF, and bivalirudin + VEGF. Following left pneumonectomy, mice in the control group received 100 μ L of normal saline via IP injection every 12 hours. Mice in the bivalirudin group were treated with IP bivalirudin at 50 mg/kg/dose every 12 hours. The VEGF group received VEGF₁₆₄ at 0.5 mg/kg/dose daily and the bivalirudin + VEGF group received both bivalirudin and VEGF treatments. Mice in all experimental groups were euthanized on POD 4 and lung volume was measured as previously described.

Pulmonary Mechanical Studies: Pulmonary mechanical measurements with the Flexivent[®] system (SCIREQ, Montreal, Canada) were done on mice in the control, heparin, and bivalirudin groups immediately before euthanasia on POD 4. After anesthetization with ketamine and xylazine, a midline neck incision was made to expose the trachea, followed by tracheotomy and insertion of a 20-gauge hollow bore needle. The animal was then connected to the Flexivent[®] system via the tracheostomy. Dynamic elastance (lung stiffness) was measured with the single frequency forced oscillation technique, which registered the animal's response to an applied sinusoidal waveform. Total lung capacity (TLC) in this study was defined as the upper limit of the expiratory limb of the pressure-volume loop and calculated with the Salazar-Knowles equation[26].

Statistical Analyses:

For *in vitro* assays, comparisons of VEGFR2 activation levels and cell densities among the different experimental conditions were performed with analysis of variance (ANOVA) and Holm-Sidak correction for multiple comparisons. Statistical analyses of lung volume, morphometric parameters, and pulmonary mechanical functions among the experimental groups were performed with ANOVA and Holm-Sidak correction for multiple comparisons. Comparisons of heparin activity, protein expression levels, plethysmographic measurements, and activity levels between the control and heparin groups were performed with Student's *t*-test. For PCR array, a difference of more than two-fold in gene expression between the control and heparin groups was considered significant. All analyses were performed on GraphPad Prism v7 (GraphPad Software, La Jolla, CA).

Results:

Heparin reduced VEGFR2 activation in the presence of the extracellular matrix and impaired proliferation of lung endothelial cells

We first sought to examine the effects of heparin on VEGF₁₆₅-mediated activation of VEGFR2 on HMVEC-L. Heparin treatment resulted in no difference in VEGFR2 activation when HMVEC-L were grown on gelatin-coated tissue culture plates (Figure 1A–B). However, on Matrigel[®]-coated tissue culture plates, the addition of heparin at 0.125, 0.25,

0.5, and 1 U/mL significantly impaired VEGFR2 activation compared to cells not treated with heparin ($P = 0.03, 0.006, 0.001$, and 0.0008 , respectively) (Figure 1C–D). These results suggested that the presence of the ECM modulated the effects of heparin on VEGFR2 activation.

To confirm the results of activation assays, proliferation of HMVEC-L treated with 10 ng/mL of VEGF₁₆₅ was assessed after 3 days of culture in the same concentrations of heparin. Compared to VEGF₁₆₅ alone, the addition of heparin at 0.125, 0.25, 0.5, or 1 U/mL resulted in a decrease in HMVEC-L density on both gelatin-coated plates ($P = 0.001, < 0.0001, < 0.0001$, and < 0.0001 , respectively) (Figure 1E) and Matrigel®-coated plates ($P = 0.04, 0.01, 0.003$, and 0.001 , respectively) (Figure 1F). The anti-angiogenic effects of heparin have been reported in human umbilical vein endothelial cells (HUVEC)[27], and a similar phenomenon was demonstrated on HMVEC-L in this study.

Systemic heparinization impaired compensatory lung growth, activity levels, and respiratory volumes after left pneumonectomy

Given the inhibitory activity of heparin on VEGFR2 activation and HMVEC-L proliferation, we speculated that systemic heparinization would impair lung growth by interfering with the interaction between VEGF and VEGFR2. To test this hypothesis, a mouse model of CLG after left pneumonectomy was used. Four experimental groups included control, heparin, VEGF, and heparin + VEGF as previously described. Systemic heparinization was first confirmed by measuring anti-factor IIa activity. Heparinized mice displayed significantly higher anti-factor IIa activity compared to control mice (0.94 ± 0.14 vs 0.32 ± 0.05 U/mL, $P = 0.004$) (Figure 2A).

Right lung volume on POD 4 was significantly lower in the heparin group compared to the control and VEGF groups (49.3 ± 1.3 vs 53.7 ± 1.0 and 59.5 ± 1.4 x $\mu\text{L/g}$, $P = 0.02$ and < 0.0001 , respectively) (Figure 2B). Compared to the heparin group, the combination of heparin and VEGF appeared to rescue lung growth (49.3 ± 1.3 vs 54.7 ± 1.8 x $\mu\text{L/g}$, $P = 0.02$) but did not stimulate growth to the same volume induced by VEGF treatment alone.

Based on the evidence of impaired CLG with systemic heparinization, we next investigated the changes produced by heparin on respiratory volume and physical activity level in live animals after left pneumonectomy. On plethysmographic studies, the heparin group displayed a trend toward reduced expiratory volume compared to control mice (1.03 ± 0.06 vs 1.21 ± 0.05 $\mu\text{L/g}$, $P = 0.06$) (Figure 2C). Although not reaching statistical significance, tidal volume ($P = 0.1$) and minute ventilation ($P = 0.1$) were also lower in the heparin group (Figure 2D and 2E). The heparinized mice similarly performed worse than the control group on respiratory flow measurements, specifically peak inspiratory flow (0.58 ± 0.03 vs 0.73 ± 0.04 mL/s, $P = 0.02$), peak expiratory flow (0.41 ± 0.03 vs 0.52 ± 0.03 mL/s, $P = 0.04$), and mid-expiratory flow (0.28 ± 0.02 vs 0.35 ± 0.02 mL/s, $P = 0.04$) (Figure 2F–H). On physical activity assessment, mice in the heparin group demonstrated a trend toward reduced walking distance (2.15 ± 0.09 vs $2.83 \pm 0.31 \times 10^3$ cm, $P = 0.09$), basic movements (1.38 ± 0.07 vs $1.84 \pm 0.18 \times 10^3$, $P = 0.06$), and fine movements (0.73 ± 0.03 vs $0.88 \pm 0.06 \times 10^3$, $P = 0.05$) (Figure 2I–K). Taken together, these results demonstrated the deleterious

effects of heparin on lung growth as evidenced by measurements of lung volume, activity levels, and plethysmography parameters after left pneumonectomy.

Systemic heparinization resulted in decreased alveolar regeneration on morphometric analyses

In order to assess changes in lung architecture, quantitative microscopy was performed using the point and intersection counting technique. Mice in the heparin group had significantly lower parenchymal volume compared to the control, VEGF, and heparin + VEGF groups (34.8 ± 2.6 vs 43.4 ± 1.8 , 47.4 ± 1.1 , and $42.5 \pm 2.2 \times \mu\text{L/g}$, $P = 0.02$, 0.001 , and 0.02 , respectively) (Figure 3A). Alveolar volume in the heparin group was also significantly lower than the remaining groups (20.6 ± 1.4 vs 26.7 ± 0.5 , 29.6 ± 0.7 , and $28.3 \pm 1.9 \times \mu\text{L/g}$, $P = 0.004$, 0.0003 , and 0.001 , respectively) (Figure 3B). Similarly, the heparin group also displayed the lowest septal surface area (14.1 ± 0.7 vs 17.2 ± 0.9 , 19.0 ± 0.6 , and $17.9 \pm 1.0 \text{ cm}^2/\text{g}$, $P = 0.02$, 0.002 , and 0.01 , respectively) (Figure 3C). On alveolar counting, the heparin group again demonstrated reduced alveolar density (2.63 ± 0.15 vs 3.07 ± 0.18 , 4.23 ± 0.07 , and $3.38 \pm 0.13 \times 10^7$ per cm^3 , $P = 0.03$, $P < 0.0001$, and $P = 0.003$, respectively) (Figure 3D). These results, which were consistent with previous functional studies, confirmed a reduction in alveolar proliferation as a result of heparin treatment.

Systemic heparinization reduced angiogenic signaling of VEGF after left pneumonectomy

To determine the effect of systemic heparin treatment on the activation pattern of lung tissue VEGFR2, we first analyzed the protein levels of phosphorylated VEGFR2 and VEGF. We found that protein levels of VEGF_{120/164} in lung tissue were significantly increased in the heparin group compared to the control group ($P = 0.0007$) (Figure 4B–C). However, the levels of VEGFR2 activation, P-Y1175-VEGFR2, were paradoxically reduced in the heparin group ($P = 0.05$) (Figure 4B–C).

To delve deeper into the physiological implications of heparin-mediated attenuation of VEGFR2 signaling, we utilized a PCR array to analyze the expression of 84 genes implicated in angiogenesis. Compared to the control group, the heparin group displayed greater than 2-fold reduction in the expression of specific groups of genes associated with P-Y1175-VEGFR2 signaling pathway. These included MEK (*Map2k2*), MAPK (*Mapk11*, *Mapk12*, and *Mapk13*), and phospholipase A2 (PLA2; *Pla2g12a*, *Pla2g2d*, *Pla2g3*, *Pla2g4a*, *Pla2g4b*, and *Pla2g5*) (Figure 4D). Only *Pik3r5*, a negative regulatory subunit of phosphoinositide 3-kinase (PI3K), was significantly upregulated in the heparin group on PCR array (Figure 4D). To further investigate this finding, we also probed for the levels of phosphorylation of Akt, a downstream effector of PI3K. Compared to control lungs, heparin-treated lungs expectedly showed decreased activation of Akt, especially at the S473 residue ($P = 0.03$) (Figure 4E–F). Taken together, the results of western blots and PCR array demonstrated a reduction in VEGFR2 activation and downstream markers of cellular proliferation and survival with systemic heparin treatment.

Bivalirudin, an alternative anticoagulant to heparin, preserved compensatory lung growth and pulmonary mechanical properties

With evidence of systemic heparinization causing impaired CLG and the resulting implications on clinical practice, bivalirudin, a direct thrombin inhibitor (DTI) and alternative anticoagulant, was investigated for its effects on lung growth. Unlike heparin, bivalirudin is not known to interact with plasma proteins. First, plasma samples of saline and bivalirudin treated mice were analyzed for coagulation functions to ensure adequate anti-coagulation. Compared to control mice, bivalirudin-treated mice showed increased prothrombin time (PT) (36.7 ± 3.7 vs 10.9 ± 0.2 seconds, $P = 0.02$) and activated partial thromboplastin time (aPTT) (83.0 ± 8.6 vs 25.4 ± 2.5 seconds, $P = 0.003$) (Figure 5A–B). Anti-factor Xa activity, which should only be elevated with heparin treatment, was not stimulated by bivalirudin (Figure 5C).

Four groups of mice underwent left pneumonectomy as previously described: control, bivalirudin, VEGF, and bivalirudin + VEGF. On POD 4, there was no difference in lung volume between the bivalirudin and the control group (Figure 5D). The VEGF and bivalirudin + VEGF group both displayed higher lung volume compared to the control group (59.5 ± 1.1 and 60.8 ± 0.9 vs 54.6 ± 1.0 $\mu\text{L/g}$, $P = 0.03$ and 0.0008 , respectively) (Figure 5D). There was no difference in lung volume between the VEGF and bivalirudin + VEGF groups.

To corroborate the results of lung volume measurements, pulmonary mechanical functions of the control, heparin, and bivalirudin groups were also determined on deeply anesthetized live animals. There was no difference in TLC between the control and bivalirudin groups. However, heparinized mice expectedly displayed reduced TLC compared to the control group (25.5 ± 0.4 vs 27.1 ± 0.5 $\mu\text{L/g}$, $P = 0.04$) (Figure 5E). Furthermore, lungs of heparinized mice showed a trend toward increased stiffness compared to the bivalirudin group (49.4 ± 2.1 vs 43.1 ± 1.5 $\text{cmH}_2\text{O/mL}$, $P = 0.06$) (Figure 5F). Unlike heparin, bivalirudin, which is not known to interact with plasma proteins such as VEGF, did not impair CLG and pulmonary mechanics after left pneumonectomy.

Discussion:

Heparin remains one of the most widely used therapies for anti-coagulation today. Results from this study raised concern with regard to the clinical use of systemic heparin in the setting of organ regeneration. Additionally, 37% of patients on ECMO were neonates with respiratory failure according to the Extracorporeal Life Support Organization's Registry 2016 report[28]. Given the established roles of VEGF in neonatal lung development[29–31], the use of heparin in this population could also impair lung growth via interfering with VEGF signaling.

In the present study, we first demonstrated the deleterious effects of heparin on VEGF₁₆₅-induced activation of VEGFR2 as well as proliferation of HMVEC-L. This effect was consistent with many other previously reported studies[14, 32]. Although the effects of heparin on HMVEC-L proliferation were marginal, it should be considered in the context of VEGF stimulation. Despite resulting in a decrease of only 20% in cell density compared to

no heparin, heparin at high concentrations completely neutralized the mitogenic effect of VEGF on HMVEC-L. Although the contribution of ECM in modulating the activity of VEGF in this study is speculative, indirect evidence of heparin-induced sequestration of VEGF has been shown in a human study where a single dose of heparin was sufficient to significantly decrease the serum level of VEGF after just 10 minutes of infusion[33]. Additionally, heparin has been demonstrated to increase the binding between VEGF₁₆₅ and the ECM[34], which could result in sequestration of VEGF and a decrease in VEGFR2 activation as seen in this study.

We then demonstrated in an *in vivo* model that the administration of heparin also impaired CLG, an angiogenesis-dependent process[35, 36]. A large dose of heparin, 500 U/kg, was used in this study to mimic the state of systemic anticoagulation in the clinical setting, where heparin level is maintained between 0.3–0.7 U/mL. Heparinized mice displayed decreased parenchymal volume, alveolar density, and alveolar septal surface area, all of which suggest a reduced capacity for gas exchange. The findings on lung volume measurements and morphometric analyses were in fact corroborated with a series of studies aimed at assessing functional outcomes, such as pulmonary mechanical studies, plethysmographic measurements, and physical activity levels. It should also be noted that despite being systemic anticoagulated, no internal bleeding was noticed at the time of organ harvest. Additionally, microscopic examination of the lung did not reveal evidence of pulmonary hemorrhage (Figure 3), which could affect parameters such as lung volume and mechanics. Collectively, these results demonstrated the damaging effects of systemic heparin treatment on lung growth after left pneumonectomy.

Although there was an increase in the tissue level of VEGF_{120/164}, P-VEGFR2 at the Y1175 position was decreased as a result of heparin administration. Given that there was no difference in the mRNA expression of *Vegfa* between the two groups (data not shown), these finding indicated that heparin-mediated modulation of VEGF is post-translational and is consistent with our hypothesis of sequestration/tethering of VEGF by cell surface or ECM bound heparin. Indirect evidence of heparin-induced sequestration of VEGF has been shown in a human study where a single dose of heparin was sufficient to significantly decrease the serum level of VEGF after just 10 minutes of infusion[33]. Additionally, heparin has been demonstrated to increase the binding between VEGF₁₆₅ and the ECM[34], which could result in sequestration of VEGF and a decrease in VEGFR2 activation as seen in this study.

Phosphorylation at the Y1175 position is a well-known initiating event that triggers the MAPK/ERK pathway of cellular proliferation[37]. In order to further confirm the effects of heparin on VEGF signal transduction, we performed a PCR array of the VEGFR2 signaling cascade. Compared to the control group, the heparin group displayed more than a 2-fold reduction in the expression of three main groups of downstream mediators: MAPK, PLA2, and NFAT and their activator, calcineurin. Furthermore, there was an increase in *Pik3r5*, a regulatory subunit of PI3K, and reduced levels of P-Akt. As Akt is a well-known pro-survival factor[38], this finding provided further proof for the adverse effects of heparin on CLG.

Given the potential inhibitory effects of heparin on lung growth, the need for anticoagulation alternatives that are not anti-angiogenic warranted further investigation. In recent years, due to the risk of heparin-induced thrombocytopenia and heparin resistance, DTIs have emerged as a safe alternative to heparin in ECMO anticoagulation[39]. In this study, we demonstrated that systemic anticoagulation with bivalirudin, a DTI, did not result in an impairment of CLG. Although there was no statistical significance, mice anticoagulated with bivalirudin displayed slightly higher lung volume compared to the control group, and the combination of VEGF and bivalirudin may even have a synergistic effect. Anticoagulation itself may augment compensatory lung growth in a fashion that is independent of VEGF. Therefore, bivalirudin and other types of anticoagulant that do not impair VEGF signaling can result in better organ growth and regeneration in patients who require systemic anticoagulation. Furthermore, the use of bivalirudin also preserved pulmonary mechanical properties. This observation therefore bears significant clinical implication and should raise concern among clinicians with regard to the use of heparin, especially in the setting of organ growth and regeneration. Bivalirudin and other direct thrombin inhibitors therefore may present a more suitable option for anticoagulation therapy that can circumvent the anti-angiogenic effects of heparin.

Acknowledgments:

The authors acknowledge Kristin Johnson of the Vascular Biology Program for her assistance with the manuscript's figures. Research funding is provided by the Boston Children's Hospital Surgical Foundation and the Vascular Biology Program at Boston Children's Hospital, the Corkin and Maher Family Fund (PDM), the National Institutes of Health Grants 5T32HL007734 (DTD and MAB) and 1F32DK104525 (GLF), and the neurodevelopmental behavior core of Boston Children's Hospital (CHB IDDRC - 1U54HD090255).

References:

1. Wardrop D, Keeling D (2008) The story of the discovery of heparin and warfarin. *Br J Haematol* 141:757–63. doi: 10.1111/j.1365-2141.2008.07119.x [PubMed: 18355382]
2. Onishi A, St Ange K, Dordick JS, Linhardt RJ (2016) Heparin and anticoagulation. *Front Biosci (Landmark Ed)* 21:1372–92
3. Kraushaar DC, Dalton S, Wang L (2013) Heparan sulfate: a key regulator of embryonic stem cell fate. *Biol Chem* 394:741–51. doi: 10.1515/hsz-2012-0353 [PubMed: 23370908]
4. Huegel J, Sgariglia F, Enomoto-Iwamoto M, et al. (2013) Heparan sulfate in skeletal development, growth, and pathology: The case of hereditary multiple exostoses. *Dev Dyn* 242:1021–1032. doi: 10.1002/dvdy.24010 [PubMed: 23821404]
5. Olczyk P, Mencner Ł, Komosinska-Vassev K (2015) Diverse Roles of Heparan Sulfate and Heparin in Wound Repair. *Biomed Res Int* 2015:1–7. doi: 10.1155/2015/549417
6. Li J, Vlodavsky I (2009) Heparin, heparan sulfate and heparanase in inflammatory reactions. *Thromb Haemost* 102:823–8. doi: 10.1160/TH09-02-0091 [PubMed: 19888515]
7. Chiodelli P, Bugatti A, Urbinati C, Rusnati M (2015) Heparin/Heparan Sulfate Proteoglycans Glycomic Interactome in Angiogenesis: Biological Implications and Therapeutic Use. *Molecules* 20:6342–6388. doi: 10.3390/molecules20046342 [PubMed: 25867824]
8. Coltrini D, Rusnati M, Zoppetti G, et al. (1994) Different effects of mucosal, bovine lung and chemically modified heparin on selected biological properties of basic fibroblast growth factor. *Biochem J* 583–90
9. Krilleke D, Ng Y-SE, Shima DT (2009) The heparin-binding domain confers diverse functions of VEGF-A in development and disease: a structure-function study. *Biochem Soc Trans* 37:1201–6. doi: 10.1042/BST0371201 [PubMed: 19909247]

10. Norrby K (2000) 2.5 kDa and 5.0 kDa heparin fragments specifically inhibit microvessel sprouting and network formation in VEGF165-mediated mammalian angiogenesis. *Int J Exp Pathol* 81:191–8 [PubMed: 10971740]
11. Chung SW, Bae SM, Lee M, et al. (2015) LHT7, a chemically modified heparin, inhibits multiple stages of angiogenesis by blocking VEGF, FGF2 and PDGF-B signaling pathways. *Biomaterials* 37:271–278. doi: 10.1016/j.biomaterials.2014.10.004 [PubMed: 25453957]
12. Casu B, Guerrini M, Guglieri S, et al. (2004) Undersulfated and Glycol-Split Heparins Endowed with Antiangiogenic Activity. *J Med Chem* 47:838–848. doi: 10.1021/jm030893g [PubMed: 14761186]
13. Koyanagi S, Tanigawa N, Nakagawa H, et al. (2003) Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochem Pharmacol* 65:173–9 [PubMed: 12504793]
14. Pisano C, Aulicino C, Vesci L, et al. (2005) Undersulfated, low-molecular-weight glycol-split heparin as an antiangiogenic VEGF antagonist. *Glycobiology* 15:1C–6C. doi: 10.1093/glycob/cwi007 [PubMed: 15329357]
15. Rezzola S, Dal Monte M, Belleri M, et al. (2015) Therapeutic Potential of Anti-Angiogenic Multitarget N,O-Sulfated E. Coli K5 Polysaccharide in Diabetic Retinopathy. *Diabetes* 64:2581–92. doi: 10.2337/db14-1378 [PubMed: 25695948]
16. Panigrahy D, Kalish BT, Huang S, et al. (2013) Epoxyeicosanoids promote organ and tissue regeneration. *Proc Natl Acad Sci* 110:13528–13533. doi: 10.1073/pnas.1311565110 [PubMed: 23898174]
17. Ding B-S, Nolan DJ, Guo P, et al. (2011) Endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization. *Cell* 147:539–53. doi: 10.1016/j.cell.2011.10.003 [PubMed: 22036563]
18. Sakurai MK, Greene AK, Wilson J, et al. (2005) Pneumonectomy in the Mouse: Technique and Perioperative Management. *J Investig Surg* 18:201–205. doi: 10.1080/08941930591004485 [PubMed: 16126631]
19. Li Y, Sun J-F, Cui X, et al. (2011) The effect of heparin administration in animal models of sepsis: a prospective study in *Escherichia coli*-challenged mice and a systematic review and metaregression analysis of published studies. *Crit Care Med* 39:1104–12. doi: 10.1097/CCM.0b013e31820eb718 [PubMed: 21317646]
20. Sakurai MK, Lee S, Arsenaault DA, et al. (2007) Vascular endothelial growth factor accelerates compensatory lung growth after unilateral pneumonectomy. *Am J Physiol Lung Cell Mol Physiol* 292:L742–7. doi: 10.1152/ajplung.00064.2006 [PubMed: 17122356]
21. Scherle W (1970) A simple method for volumetry of organs in quantitative stereology. *Mikroskopie* 26:57–60 [PubMed: 5530651]
22. Ochs M, Mühlfeld C (2013) Quantitative microscopy of the lung: a problem-based approach. Part 1: basic principles of lung stereology. *Am J Physiol Lung Cell Mol Physiol* 305:L15–22. doi: 10.1152/ajplung.00429.2012 [PubMed: 23624789]
23. Mühlfeld C, Ochs M (2013) Quantitative microscopy of the lung: a problem-based approach. Part 2: stereological parameters and study designs in various diseases of the respiratory tract. *Am J Physiol Lung Cell Mol Physiol* 305:L205–21. doi: 10.1152/ajplung.00427.2012 [PubMed: 23709622]
24. Weibel ER, Gomez DM (1962) A principle for counting tissue structures on random sections. *J Appl Physiol* 17:343–8 [PubMed: 14005589]
25. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408. doi: 10.1006/meth.2001.1262 [PubMed: 11846609]
26. Salazar E, Knowles JH (1964) An analysis of pressure-volume characteristics of the lungs. *J Appl Physiol* 19:97–104 [PubMed: 14104296]
27. Collen A, Smorenburg SM, Peters E, et al. (2000) Unfractionated and low molecular weight heparin affect fibrin structure and angiogenesis in vitro. *Cancer Res* 60:6196–200 [PubMed: 11085545]

28. Thiagarajan RR, Barbaro RP, Rycus PT, et al. (2017) Extracorporeal Life Support Organization Registry International Report 2016. *ASAIO J* 63:60–67. doi: 10.1097/MAT.0000000000000475 [PubMed: 27984321]
29. Thébaud B, Ladha F, Michelakis ED, et al. (2005) Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. *Circulation* 112:2477–86. doi: 10.1161/CIRCULATIONAHA.105.541524 [PubMed: 16230500]
30. van der Horst IWJM, Rajatapiti P, van der Voorn P, et al. Expression of hypoxia-inducible factors, regulators, and target genes in congenital diaphragmatic hernia patients. *Pediatr Dev Pathol* 14:384–90. doi: 10.2350/09-09-0705-OA.1
31. Schmidt AF, Gonçalves FLL, Regis AC, et al. (2012) Prenatal retinoic acid improves lung vascularization and VEGF expression in CDH rat. *Am J Obstet Gynecol* 207:76.e25–76.e32. doi: 10.1016/j.ajog.2012.04.025
32. Kim J, Al-Hilal TA, Chung SW, et al. (2015) Antiangiogenic and anticancer effect of an orally active low molecular weight heparin conjugates and its application to lung cancer chemoprevention. *J Control Release* 199:122–131. doi: 10.1016/j.jconrel.2014.12.015 [PubMed: 25523032]
33. East MA, Landis DI, Thompson MA, Annex BH (2003) Effect of single dose of intravenous heparin on plasma levels of angiogenic growth factors. *Am J Cardiol* 91:1234–6 [PubMed: 12745108]
34. Sack KD, Teran M, Nugent MA (2016) Extracellular Matrix Stiffness Controls VEGF Signaling and Processing in Endothelial Cells. *J Cell Physiol* 231:2026–2039. doi: 10.1002/jcp.25312 [PubMed: 26773314]
35. Jancelewicz T, Grethel EJ, Chapin CJ, et al. (2010) Vascular Endothelial Growth Factor Isoform and Receptor Expression During Compensatory Lung Growth. *J Surg Res* 160:107–113. doi: 10.1016/j.jss.2008.10.007 [PubMed: 19535097]
36. Zhang Q, Bellotto DJ, Ravikumar P, et al. (2007) Postpneumonectomy lung expansion elicits hypoxia-inducible factor-1 α signaling. *Am J Physiol Lung Cell Mol Physiol* 293:L497–504. doi: 10.1152/ajplung.00393.2006 [PubMed: 17513452]
37. Takahashi T, Yamaguchi S, Chida K, Shibuya M (2001) A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC- γ and DNA synthesis in vascular endothelial cells. *EMBO J* 20:2768–2778. doi: 10.1093/emboj/20.11.2768 [PubMed: 11387210]
38. Franke TF, Kaplan DR, Cantley LC (1997) PI3K: downstream AKTion blocks apoptosis. *Cell* 88:435–7 [PubMed: 9038334]
39. Raiten JM, Wong ZZ, Spelde A, et al. (2016) Anticoagulation and Transfusion Therapy in Patients Requiring Extracorporeal Membrane Oxygenation. *J Cardiothorac Vasc Anesth* doi: 10.1053/j.jvca.2016.08.011

Figure 1: Heparin reduces HMVEC-L proliferation and VEGFR2 activation in the presence of extracellular matrix

Heparin did not affect VEGF₁₆₅-mediated activation of VEGFR2 on human microvascular lung endothelial cells (HMVEC-L) grown on gelatin-coated tissue culture plates (A–B). Activation of VEGFR2 was decreased with heparin in a dose dependent manner when HMVEC-L were grown on Matrigel®-coated plates (C–D). All experiments were done in duplicates. Heparin impaired HMVEC-L proliferation in the presence of VEGF₁₆₅ at 10 ng/mL on both gelatin and Matrigel®-coated plates in a dose dependent manner (E–F). Cell viability was assessed with a tetrazolium assay after 3 days of incubation. All experiments

were done in quadruplicates. OD: optical density. Results are expressed as mean $\pm$ SE. Kd: dissociative constant. *: P 0.05; **: P 0.01; **** P 0.0001 compared to heparin 0 U/mL. ++: P 0.01; +++: P 0.001 compared to heparin 0.25 U/mL.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2: Heparin impaired compensatory lung growth, plethysmographic measurements, and activity levels in mice after left pneumonectomy

Systemically heparinized mice had higher plasma heparin activity levels compared to control mice as assessed by anti-factor IIa activity assays (A). The heparin group displayed the lowest lung volume among the 4 experimental groups on post-operative day (POD) 4. Compared to the control group, the heparin group demonstrated lower plethysmographic parameters, including expiratory volume (C), tidal volume (D), minute ventilation (E), peak inspiratory flow (F), peak expiratory flow (G), and mid-expiratory flow (H). Systemically heparinized mice also demonstrated reduced walking distance (I) and movement counts (J–

K) on open field studies. Statistical analyses of lung volume among the experimental groups were performed with ANOVA and Holm-Sidak correction for multiple comparisons. Results are expressed as mean $\pm$ SE. *: $P \leq 0.05$; **: $P \leq 0.01$; **** $P \leq 0.0001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3: Heparin impaired compensatory lung growth as demonstrated by morphometric analyses of lung tissues

Systemically heparinized mice displayed the lowest parenchymal volume (A), alveolar volume (B), septal surface area (C), and alveolar density (D) among the 4 experimental groups on POD 4 after left pneumonectomy. Micrographs at 200X magnification of hematoxylin and eosin-stained control (E), heparin (F), VEGF (G), and heparin + VEGF (H) lung sections are shown. Results are expressed as mean $\pm$ SE. *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 4: Heparin impaired VEGFR2 activation and reduced VEGF angiogenic signaling after left pneumonectomy in mice
 VEGFR2 signaling cascade was illustrated (A). Heparin increased lung tissue levels of VEGF_{120/164} but decreased activation of VEGFR2 (B and C). Polymerase chain reaction (PCR) array of VEGFR2 signaling cascade confirmed the reduction of downstream effectors such as mitogen-activated protein kinase kinase (MEK), mitogen-activated protein kinase (MAPK), phospholipase A2 (PLA2), nuclear factor of activated T-cells (NFAT), and calcineurin (D). Only *Pi3kr5*, which encodes a negative regulatory subunit of phosphoinositide 3-kinase (PI3K), was up-regulated. Confirmation with western blot

revealed reduction of Akt phosphorylation at residue S473 in the heparin-treated mice (E and F). Results are expressed as mean $\pm$ SE. *: $P = 0.05$; **: $P = 0.01$; *** $P = 0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5: Bivalirudin did not affect compensatory lung growth and preserved pulmonary mechanical functions after left pneumonectomy

Bivalirudin, a direct thrombin inhibitor that does not interact with plasma proteins, was an effective anticoagulant in mice as demonstrated by increased prothrombin time (PT) and partial thromboplastin time (PTT) (A and B). Anti-factor Xa activity, which is only increased with heparin treatment, was not affected by bivalirudin (C). There was no difference in lung volume on POD 4 between the bivalirudin-treated and control mice (D). The combination of VEGF and bivalirudin also did not reduce the effects of VEGF on accelerating lung growth. On pulmonary mechanical studies, bivalirudin preserved total lung

capacity (E) and elastance/stiffness (F) while heparinized mice performed worse on those measurements. Results are expressed as mean $\pm$ SE. *: $P \leq 0.05$; **: $P \leq 0.01$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript