Vascular endothelial growth factor in developing ovine lung: endogenous expression and effects of exogenous administration on respiratory syncytial virus infection

Alicia Kathleen Olivier

Iowa State University

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Vascular endothelial growth factor in developing ovine lung: endogenous expression and effects of exogenous administration on respiratory syncytial virus infection

by

Alicia Olivier

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Program of Study Committee:
Mark R. Ackermann, Major Professor
Jesse M. Hostetter
Doug E. Jones
Randy E. Sacco
Ricardo F. Rosenbusch

Iowa State University
Ames, Iowa
2010

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ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis in children with nearly all children infected by two years of age. Preterm infants and infants with cardiopulmonary compromise or immunodeficiency are at increased risk for severe RSV disease. However, a high percentage of hospitalizations due to severe RSV disease occur in otherwise healthy infants. Severe RSV disease is characterized by bronchiolitis and airway obstruction secondary to sloughed epithelial cells, cellular debris and inflammatory cells that can partially occlude the airway lumen. There is currently no licensed vaccine to prevent RSV infection.

The purpose of this study was to develop a model of RSV infection in the newborn lamb using a human strain of RSV, strain A2. The pulmonary pathology and cellular localization of antigen was determined, in addition to the viral effect on the expression of epithelial innate immune genes, surfactant proteins A and D (SP-A and SP-D respectively) and sheep beta-defensin-1 (SBD-1). Pulmonary lesions were characterized by suppurative bronchiolitis with multifocal alveolar consolidation and peak pulmonary lesions at day 6 post-infection. RSV infection increased expression of SP-A and SBD-1, which demonstrates alteration of innate immune responses caused by RSV A2. Using this model, lambs were pretreated with exogenous human recombinant vascular endothelial growth factor (rhVEGF) prior to RSV infection. VEGF is a known endothelial mitogen, but also plays an important pulmonary role in surfactant protein production, epithelial cell proliferation, and epithelial cell survival. VEGF administration prior to RSV infection decreased both viral load and pulmonary pathology at peak
infection – 6 days post-infection. In addition, VEGF increased the expression of SP-A and SBD-1 showing that VEGF can induce expression of important epithelial innate immune genes. VEGF pretreatment altered epithelial cell proliferation in RSV infected animals at day 4 post-infection. In the final part of this study, the effect of ontogeny on pulmonary endogenous VEGF expression was investigated. The results of this study demonstrate that the expression of major VEGF isoforms are differentially regulated with high pulmonary VEGF mRNA expression in prenatal lamb lung and low expression in adult lung. However, mRNA expression diverged from the protein profile with low VEGF protein expression in prenatal lung and high VEGF protein expression in adult lung, demonstrating tight translational regulation to maintain appropriate VEGF concentrations during development.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation describes the development and characterization of a lamb model of human Respiratory Syncytial Virus (RSV) infection, the effect of recombinant human vascular endothelial growth factor (rhVEGF) pretreatment on RSV infection and the effect of ontogeny on expression of endogenous vascular endothelial growth factor (VEGF) and VEGF receptors. This dissertation is divided into 5 chapters that include a General Introduction, three manuscripts that have been submitted for publication or published, and a General Conclusion. Chapter 1 provides a general review of human RSV infection, the lamb as an animal model for RSV disease, innate pulmonary immunity and a brief overview of the pulmonary functions of VEGF. Chapter 2 characterizes the pulmonary pathology of RSV infection in the neonatal lamb using a human strain of RSV and alterations in epithelial innate immune gene expression. Chapter 3 describes the effect of rhVEGF pretreatment on viral load, pulmonary pathology and epithelial innate immune gene expression in the neonatal lamb model of RSV infection characterized in Chapter 2. Chapter 4 describes the developmental pulmonary expression and cellular distribution of endogenous VEGF and VEGF receptors prenatally to adulthood. Chapter 5 includes concluding remarks and future study directions.
Literature Review

Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is a single-stranded RNA virus in the Paramyxoviridae family. RSV is the leading cause of infant bronchiolitis with approximately 20% of infants exhibiting bronchiolitis in their first year of life with almost 100% infection by 2 years of age [1]. Children often become reinfected throughout life, but the initial RSV infection is usually most severe and most likely to involve the lower respiratory tract [1]. The World Health Organization estimates that there are 64 million infections every year with 160,000 deaths worldwide [2]. The majority of cases are relatively mild, however there are populations at increased risk to develop severe pulmonary disease leading to hospitalization, mechanical ventilation and even death [3]. Infants at increased risk include premature infants and infants with chronic lung disease of prematurity, congenital heart disease and immune deficiency [4-6].

RSV – Structure

The RSV genome is comprised of 10 genes that encode 11 viral proteins. There are two nonstructural genes (NS1 and NS2) and 9 structural genes (N, P, M, SH, G, F, M2-1, M2-2 and L). The attachment (G) and fusion (F) glycoproteins are outer membrane proteins that participate in viral binding and entrance into target cells [2]. The G protein enables cell surface binding via surface glycosaminoglycans [7]. In addition, the G protein binds the fractalkine chemokine receptor (CX3CR1) and mimics the chemokine fractalkine [8]. The F protein enables virus entry by directing the fusion of
the virion envelope with the plasma membrane of the target cell. The F protein may also initiate apoptosis by p53-dependent programmed cell death and result in epithelial shedding, airway obstruction and inflammation [9]. The G and F glycoproteins are the only virus neutralization antigens. The SH glycoprotein plays a role in immune modulation and has been shown to decrease TNF-α signaling and inhibit cell death following infection [10]. The nucleocapsid N protein reduces cellular detection of RNA by encapsidating the positive-strand replicative intermediate and the genomic RNA [11]. The L protein is the major polymerase subunit and the P phosphoprotein is a cofactor in RNA synthesis [12]. The M2-1 and M2-2 proteins are involved in transcription and transcriptional regulation [13, 14]. The matrix M protein is found along the surface of the inner envelope and plays a role in virion morphogenesis [15]. The NS1 and NS2 proteins are not packaged significantly within the virion and are nonessential accessory proteins. NS1 and NS2 decrease type I interferon pathways and modulating the host response to infection [16]. Interestingly, recombinant RSVs that contain mutations in the NS1 and NS2 proteins replicate poorly in humans showing their important role in altering the host immune response [17].

There are two major RSV groups, A and B, based on antigenic and molecular analysis [18]. Several reports show that children infected with group A are significantly older than those infected with group B with a higher frequency of fever [19]. However, other studies show no difference between the two groups and comparison is often difficult due to intercurrent issues such as prematurity and underlying conditions [20, 21].

**RSV – Transmission and Replication**
Inoculation of RSV occurs via aerosol or direct contact via the mucosa of the nose or eye [22]. Replication occurs within the epithelium of the nasopharynx for approximately 4-5 days. Spread to the lower respiratory tract can follow the replication period. In the lower respiratory tract, RSV targets epithelial cells of the bronchioles and type I pneumocytes that line the alveoli [23]. Other cells that can be infected include macrophages and dendritic cells [24].

Upon infection, the nucleocapsid and polymerase are delivered to the host cell cytoplasm where the genome is transcribed and replicated. The ten viral genes are flanked by gene-start (GS) and gene-end (GE) sequences which function to control the polymerase throughout transcription. The polymerase often dissociates from the template at the gene junctions, therefore the genes at the 3’ end of the genome are transcribed more frequently [25]. To replicate the genome the polymerase does not respond to the GE and GS signals and results in the transcription of a positive sense complement of the genome (antigenome). The antigenome can then be used to make genome-sense RNA that will be encapsidated with virus nucleoprotein as synthesis occurs [2]. Virions bud through the plasma membrane to acquire a lipid envelope [24].

**RSV – Populations at increased risk**

The risk of severe RSV disease is increased by a myriad of factors including premature birth, low birth weight, large family size, male gender, lack of breast feeding, exposure to passive cigarette smoke and birth approximately 4 months before the peak of the RSV season [26]. Other infants at high risk include those with cardiovascular disease [27], neuromuscular diseases [28] and immune deficiency syndromes [3, 29]. RSV
disease related mortality rates are significantly increased in high-risk infant populations [30]. RSV can also cause severe disease in adults, especially patients with underlying cardiopulmonary disease, the elderly and immunocompromised individuals [31, 32]. In older adults, RSV is associated with the exacerbation of chronic obstructive pulmonary disease [33]. The Centers for Disease Control and Prevention estimate that 10,000 deaths annually are attributed to RSV infection in adults over the age of 65 years [34].

RSV – Clinical Signs and Pulmonary Pathology

Clinical signs secondary to RSV infection include cough, rhinorrhea, and low grade fever. Infection of the lower airway causes bronchiolitis with wheezing and increased airway resistance. 50% or more of hospitalized infants with lower respiratory infection have wheezing episodes following resolution of clinical signs. Wheezing in these individuals could persist until children are 11 years of age with loss of this effect by 13 years [35]. There is controversy regarding RSV’s role in asthma development, although RSV is thought to induce a Th2-type response that promotes the development of asthma later in life. Hypoxia secondary to pneumonia can also be an associated sign in RSV infected infants [24].

Histopathology findings attributed to RSV infection include bronchiolar epithelial necrosis with epithelial sloughing into the airway lumen [36]. Airway obstruction results from occlusion of the airway with cellular debris, mucus, neutrophils and occasional macrophages. Multifocally within the bronchiolar epithelium are areas of epithelial proliferation and syncytial cell formation [23]. Additionally, within the bronchiolar lumen and bronchiolar epithelium are infiltrates of macrophages, neutrophils. The
bronchiolar subepithelial area and adventitia are infiltrated by T, B cells and occasional plasma cells, depending on the stage of disease [24]. The pathogenesis of the lesions will be discussed in a later section.

RSV: Therapeutics and therapeutic targets

At this time there are no effective vaccines for RSV infection. Palivizumab (Synagis), a humanized monoclonal antibody, is the only approved prophylactic treatment for infants at high risk. Palivizumab is directed against the F surface glycoprotein. Palivizumab reduces the frequency of severe RSV disease by 50% in children at high risk and therefore decreases the development of bronchiolitis and subsequent hospitalization [37, 38]. A formalin inactivated vaccine formulated in the 1960’s in the United States failed to protect children against infection and actually enhanced disease during natural infection in infants and toddlers. Two infants that were immunized with the vaccine died upon subsequent infection. High titers of RSV were recovered from these infants [39]. In vivo studies in animals models have shown that unlike natural infection, formalin inactivated RSV leads to development of a Th2 memory response [40, 41]. Clinical manifestations of enhanced disease included bronchoconstriction and severe pneumonia. Pulmonary histology of patients with enhanced disease have increased peribronchiolar eosinophils [42] and deposits of antibody complexed with virus in affected tissue [43]. Given the outcome of this vaccine there have been huge efforts to understand vaccine enhanced disease and research to determine the best method for vaccination against RSV.
Pathogenesis of RSV disease

Why some individuals infected by RSV develop severe bronchiolitis and airway occlusion while others do not is a key question in understanding the pathogenesis of RSV disease and in the development of future therapeutic targets. There are a wide variety of host factors and viral factors that contribute to disease outcome. The host and viral factors that contribute to disease will be discussed in more detail in the following sections.

Recognition of RSV by the host and activation of the innate immune response

The activation of the host innate immune response during RSV infection depends on complex signaling events. Pattern recognition receptors (PRRs) detect viral binding and entry into epithelial cells, thereby stimulating responses that trigger the production of cytokines, chemokines and other antimicrobial products [44]. The most well-known PRRs are the Toll-like receptors (TLRs) that sense a wide variety of microbial ligands at the cell surface or within intracellular endosomes. A wide variety of TLRs have been reported to activate innate immune responses in RSV infection including TLR2 [45, 46], TLR4 [47], TLR3 [48], TLR6 and TLR7. A study using TLR4-deficient mice showed impaired RSV clearance, impaired IL-12 production and decreased natural killer cell function [47], showing the importance of TLRs in triggering anti-viral responses. Although the overall effect of TLR4 on disease pathogenesis is unclear, a recent report demonstrated TLR4 polymorphisms in high-risk infants were linked to symptomatic RSV disease [49]. Other cells, such as immature dendritic cells can sense virus through toll like receptors (TLRs), which can trigger T- and B-cell responses [50]. Additionally, viral
replication products are also recognized by cytoplasmic sensing pathways, including retinoic acid induced gene (RIG)-I and melanoma differentiation antigen (MDA)-5 proteins. RIG-I is a highly inducible cytoplasmic RNA helicase that induces the expression of Type I interferons and other antiviral mediators after binding double-stranded RNA [51]. MDA5 can be activated by long double stranded RNA generated during infection to drive IFN responses [52].

**Chemokines and cytokines**

Chemokines are involved in chemotaxis of recruited immune cells at the site of infection. Chemokines are classified into three groups termed C, C-C, and C-X-C based on the number and spacing of conserved NH$_2$-terminal cysteine residues. In addition there is a fourth group, the C-X$_3$.C group which is composed of large, membrane bound glycoproteins [53]. The role of chemokines and their effect on RSV disease severity is still unclear. Nasal and tracheal aspirates from RSV infected infants have significant expression proinflammatory cytokines including, regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP-1$\alpha$) and monocyte chemotactic protein (MCP-1) [54, 55]. The expression of these chemokines correlates to the number of leukocytes present in the sample [56]. MIP-1$\alpha$ appears to be an important initiator of the inflammatory response in RSV infection. MIP-1$\alpha$ knockout mice develop less severe pulmonary inflammation when infected with RSV as compared to wild-type mice [57]. In addition, polymorphisms of the MIP-1$\alpha$ receptor have been reported with RSV bronchiolitis in infants [58].
Epithelial cells play an important role in promoting and regulating inflammatory responses. Proinflammatory cytokines produced by RSV infected epithelial cells include; IL-6, IL-8, TNF-α, IFN-α and IFN-β [59]. In addition, growth factors such as GM-CSF and G-CSF are produced by RSV infected epithelial cells [60]. Higher levels of both IL-6 and IL-8 correlate with an increased disease severity [61]. Cytokines associated with adaptive responses will be discussed further in the adaptive immune response section.

Numerous viruses alter or modify expression of chemokine and cytokine responses and thereby alter host responses. A recent study examined the expression of suppressor of cytokine signaling (SOCS) protein in vitro during RSV infection in human bronchiolar epithelial cells. SOCS proteins negatively regulate chemokine and cytokine responses [62]. This study found that the RSV G glycoprotein upregulated SOCS expression resulting in a decrease in type I IFNs and interferon stimulated gene (ISG) expression [63]. A decrease in type I IFNs facilitates viral replication due to reduced expression of downstream products such as the GTPase Mx1 (myxovirus resistance 1) protein, ISG15 (IFN-stimulated protein of 15 kDa), RNaseL (ribonuclease L) and PKR (protein kinase R) [64]. Mx GTPases are located near the smooth endoplasmic reticulum and survey exocytic events and trap essential viral components to prevent early viral replication [65]. ISG15 prevents virus-mediated degradation of IFN transcription factors thereby increasing IFNβ expression [66]. Additionally, ISG15 acts as a cytokine to modulate the immune response [67]. RNaseL is activated through by-products generated by OAS proteins. OAS proteins synthesize 2’,5’-linked phosphodiester bonds to polymerize ATP into oligomers of adenosine. Activated RNaseL mediates RNA
PKR proteins respond to environmental stresses in order to regulate protein synthesis. PKR phosphorlyates EIF2α which prevents the recycling of GDP and stops translation [64].

IFN-α and IFN-β are Type I interferons. Type I IFNs are regulated by transcription factors such as interferon regulatory factor 3 (IRF-3) and nuclear factor-κB (NF-κB) [69]. As previously mentioned, Type I interferons can also be induced by TLR activation, specifically TLR3 in RSV infection [11]. In addition Type I interferons can be produced by dendritic cells and macrophages [70]. Type I interferons play a role in antiviral host defense in addition to promoting interferon-stimulated genes (ISG), natural killer (NK) cells, and dendritic cell maturation [71]. As indicated, RSV proteins modulate the immune response to better enable viral replication and survivability. The nonstructural proteins, NS1 and NS2 can also down-regulate type I IFNs by a variety of mechanisms that inhibit the formation of downstream signaling complexes such as ISG15, Mx1, RnaseL and PKR [72].

**Inflammatory cells**

Chemokines released by airway epithelial cells recruit leukocytes from the circulation to the site of infection. Neutrophils are the first inflammatory cell to arrive, between days 1-6 days post-infection. Lymphocytes infiltrate between days 4-11 with the greatest number of lymphocytes at day 11 [73]. IL-8 is the primary neutrophil chemoattractant secreted by epithelial cells. Neutrophils are present within the bronchiolar lumen and epithelium and have the ability to adhere to epithelial cells thereby enhancing epithelial damage [74, 75].
Alveolar macrophages are critical for removal of debris, apoptotic cells and also to remove opsonized material. A recent study examined the role of alveolar macrophages in RSV disease using New Zealand black mice, which have decreased number and function of alveolar macrophages. These RSV-infected mice have increased viral load and airway occlusion. Airway occlusion is a key feature of RSV disease, indicating the importance of macrophages in the removal of this cellular debris [76].

Natural killer (NK) cells exhibit nonspecific cytotoxicity against cells that express stress markers. NK cells produce IFN-γ and regulate activity of other innate and adaptive immune responses [77]. Although the role of NK cells in viral infection is important, NK cell activity in RSV infection is largely unknown. NK cells have not been detected in autopsy studies from infants with fatal RSV bronchiolitis [78].

**Adaptive immune responses to RSV**

The humoral immune response to RSV infection results in production of anti-RSV antibodies of the IgM and IgG isotype in addition to secretory IgA in the respiratory tract. Antibody responses are primarily against the surface glycoproteins, G and F, with virus neutralization being mediated predominantly by anti-F glycoprotein antibodies. Primary antibody responses to RSV peak approximately 3-4 weeks post-infection and then decline. Strong antibody responses occur within 2-3 days following re-infection. Antibody responses are not completely protective as individuals with high neutralizing antibody titers are often reinfected with RSV. Therefore a variety of other host factors determine whether the host clears the infection.
Cell mediated immune responses play a vital role in removal of virally infected cells. Viral replication lasts for weeks to months in immunocompromised individuals infected with RSV. Infants with RSV infection have weak antiviral cytotoxic responses [78]. Pulmonary tissue from fatal infant RSV infections failed to show infiltration of CD4+ or CD8+ lymphocytes. Why these infants lacked cytotoxic lymphocytes is not completely understood, but may be related to immature lymphocytes or alterations with antigen presentation by immature dendritic cells [79]. Cytokines associated with T lymphocytes including, IL-2, IL-4, IL-5, IL-13, IL-17 and IFNγ are present at low concentrations during the peak of infection in infants. This indicates that important cytotoxic antiviral responses are present at only low levels or lacking in infants [78].

The neonatal immune system

The peak hospitalization for RSV disease is approximately 2 months of age. RSV induces disease in infants earlier than other respiratory viruses, such as influenza and rhinovirus [80]. There are a variety of factors that contribute to disease pathogenesis in this young age group, including pulmonary anatomy (smaller airways), immunologic immaturity and maternal immunity. Smaller airways are more prone to occlusion. Immune responses are somewhat dampened compared to those in older children. For example, young infants have fewer number of lymphocytes in peripheral lymphoid tissue as compared to adults [24]. In addition, neonatal dendritic cells are less efficient in promoting T-cell proliferation in response to antigen stimulation and produce fewer cytokines following RSV infection [81]. Cellular cytotoxic responses against RSV were detected in less than 40% of infants under 5 months of age compared to 65% in children.
6 months to 2 years [82]. *In vitro* studies using alveolar macrophages from infants exhibit multiple deficiencies in function including, impaired chemokine release, oxidative burst and phagocytosis [83].

It is well documented that young infants mount a poor antibody response to viral infection or immunization [84, 85]. In fact, the serum and mucosal antibody response to the G and F glycoproteins of young infants to RSV infection is approximately 15-25% that of older infants and children [86]. The reason for the decreased antibody responses is due to both high levels of maternally acquired RSV serum antibodies as well as immunologic immaturity. Given these less effective acquired immune responses, the innate immune response is vitally important in controlling initial infection.

**Pulmonary Host Defense and Innate Immunity**

The lung is constantly exposed to inhaled particulates, pathogens and allergens. Therefore, an extensive network of defense mechanisms are necessary to protect the lung from damage. These mechanisms include: the epithelial barrier, mucociliary apparatus, antimicrobial products present with the surfactant layer and airway surface liquid, and alveolar macrophages. Epithelial cells also produce a wide variety of products that have inflammatory and immunoregulatory functions. The innate host pulmonary defense is composed of many systems that serve to: 1) detect pathogens and remove them, 2) signal immune cells to the site and 3) provide responses that will stimulate an optimal adaptive immune response. The following sections will describe the functions of the epithelial
derived products, surfactant proteins and β-defensins, in pulmonary immunity and their importance in RSV infection.

**Antimicrobial peptides - Defensins**

Pulmonary antimicrobial peptides include members of the defensin and cathelicidin classes. Antimicrobial peptides have broad-spectrum activity against Gram-positive and Gram-negative bacteria, viruses, fungi and yeast. Three families of defensin peptides have been identified including the α-defensins, β-defensins and σ-defensins. In humans, the α-defensins are expressed in Paneth cells of the intestine, azurophilic granules of neutrophils, and other epithelial surfaces such as the vagina, cervix and fallopian tubes [87]. The β-defensins are present in mucosal epithelial cells, mucosal secretions and granulocytes. The σ-defensins have been identified in leukocytes from nonhuman primates - rhesus macaque and olive baboon [88, 89]. Cathelicidins are antimicrobial peptides that are derived from proteins. In humans, only one cathelicidin has been characterized, LL-37, which is derived from the C-terminal end of the human CAP18 protein (hCAP18) [90]. The cathelicidin present in sheep is SMAP that is present within leukocytes (sheep myeloid antimicrobial peptide) [91].

**β-Defensin Structure**

β-defensins are cationic peptides with a broad-spectrum of antimicrobial activity. They are secreted at a wide variety of epithelial surfaces including the skin, gingiva, intestine, urogenital tract, kidney and respiratory epithelium [92]. β-defensins have three disulphide bonds between six conserved cysteine residues, which results in a triple-
stranded β-sheet structure and a β-hairpin loop containing cationic charges [87]. β-defensins are small – the preprotein is approximately 65 amino acids, which is then cleaved to form a mature peptide that is approximately 45 amino acids [93]. In addition to their small size, they have a high concentration of cationic residues (arginine, lysine, histidine) which enable them to interact and alter cellular membranes of microbial agents. The cholesterol content of mammalian cells appear to protect host cells from beta defensin-mediated damage [94].

β-Defensin Expression

The β-defensin gene has two exons that encode for a single gene product. In humans, there are five β-defensins produced by epithelial cells; human β-defensin (HBD)-1 [95], HBD-2 [96], HBD-3 [97], HBD-4 and HBD-5 [98]. HBD-1, -2, and -3 are produced by epithelial cells of the lung. HBD-1 expression in the lung is constitutive whereas HBD-2 and HBD-3 are inducible by pro-inflammatory stimuli induced by bacteria, viruses and microbial products [99, 100]. Inflammatory mediators which activate toll like receptors (TLRs) can upregulate β-defensin expression. Airway epithelial cells expresses TLR1 through TLR6 and TLR9 [101], therefore a wide variety of stimuli from bacterial LPS to viral RNA can induce β-defensin expression [102]. In addition, expression of β-defensins is developmentally regulated in late gestation and throughout the neonatal period, which can lead to increased susceptibility to respiratory tract infection in younger animals that have lower expression [103]. β-defensins have a critical role in host defense either as constitutive or inducible components of the epithelial barrier [92].
The nomenclature of β-defensin genes is rather confusing. The genes were originally numbered sequentially based on discovery; however the human gene name was changed to the gene name of the mouse ortholog, if an ortholog existed. If an orthologous gene had not been identified, then the human gene was assigned a number greater than 100. Therefore the human gene DEFB2 encoding the peptide HBD-2 was changed to DEFB4 due to its orthology to mouse Defb4 [93]. Murine β-defensins (MBD) including MBD-1, -2, -3, and -4 have been detected in mucosal tissue with antimicrobial properties similar to human β-defensins [104]. Recently, MBD-14 was discovered to be the orthologue of HBD-3 [105] and exhibits similar antimicrobial and chemotactic activity to that of HBD-3. Two ovine β-defensins have been discovered, sheep beta-defensin (SBD) -1 and SBD-2, both of which are highly expressed in the lung, in addition to other tissues such as intestine [103]. SBD-1 is inducible in parainfluenza-3 and RSV infection [106, 107].

**β-Defensin Activities**

**Antimicrobial**

β-defensin antimicrobial activity is predominantly mediated by electrostatic binding of the cationic peptide to the outer surface of the pathogen with insertion of a peptide into the cytoplasmic membrane, resulting in cellular leakage [87].

The antiviral effects of defensins are attributed to both direct interactions with the virus and through interaction with the target cell. β-defensins have been shown to have anti-viral activities on several viruses including HIV-1, vaccinia virus, adenovirus,
rhinovirus, herpes simplex virus, RSV and influenza virus [98, 108]. In influenza virus infection, HBD-3 inhibits hemagglutinin-mediated fusion to prevent infection of the host cell [109]. The antiviral activity of defensins has been most studied in HIV infection. Studies show that HBD-2 and HBD-3 have anti-HIV activities through direct interaction with the virus and also by indirectly altering the target cell. Interestingly, HBD-2 inhibits the formation of early reverse-transcribed HIV DNA products but does not affect cell-cell fusion [110]. A recent study demonstrated in vitro and in vivo induction of β-defensin expression following RSV infection [111]. The induction was dependent on NF-κB activation of tumor necrosis factor (TNF). During RSV infection TNF is produced by epithelial cells and macrophages. Increased TNF could therefore increase HBD-2 production. Also this study demonstrated that HBD-2 inhibited RSV cellular entry, with loss of antiviral activity following HBD-2 silencing using siRNA [111].

Chemotaxis Activity and Immune Regulation by Beta Defensins

β-defensins have chemotactic activity for monocytes, immature dendritic cells, and T cells. In addition, β-defensins can induce cytokine production by monocytes and epithelial cells.

β-defensins have a role linking innate and adaptive immune responses [112]. Mouse β-defensin 2 is chemotactic for immature dendritic cells and memory T cells through the chemokine receptor CCR6 [105]. HBD-3 and -4 induce mast cell chemotaxis and degranulation [113].

Summary
In summary, β-defensins have important anti-viral activities in the lung. β-defensins are developmentally regulated which may account for decreased innate immune responses in young individuals, making them more susceptible to RSV and other infections.

**Surfactant Proteins A and D - Collectins**

The primary function of the lung is to adequately maintain oxygenation of the blood for tissue distribution. For gas exchange to occur in the lung the alveoli must remain inflated throughout respiration, this is especially important during expiration. Surfactant proteins function to prevent alveolar collapse by reducing the surface tension. Surfactant is composed primarily of the phospholipid, dipalmitoyl phosphatidylcholine (DPPC), which comprises approximately 40-45% of the total surfactant mass. Other lipids including phosphatidylcholines, phosphatidylglycerol and cholesterol make up the remaining surfactant mass. Surfactant proteins B and C (SP-B and SP-C) are important in reducing surface tension and co-assemble with other surfactant lipids in the type II epithelial cell. SP-A and SP-D are secreted by a different pathway and serve varying functions which will be detailed later [114, 115]. Following surfactant secretion, the surfactant is organized into structures called tubular myelin, mono- and multi-layered sheets and vesicles [115]. SP-B is the only essential surfactant protein in reducing surface tension, however SP-A and SP-D play an important role in surfactant homeostasis [116, 117]. SP-A and SP-D are members of the collectin family and are primarily involved in innate immune defense [118]. Recent studies show SP-C also interacts with infectious agents and pattern recognition molecules, and is therefore also involved in pulmonary innate immunity [117].
Collectins are large, oligomeric glycoproteins with four domains; a cysteine-containing N-terminal portion, a collagen-like domain, an α-helical coiled neck and a C-type lectin domain or carbohydrate recognition domain (CRD). With SP-A the subunits trimerize to form a single subunit that are then linked by disulfide bonds into oligomers of up to six subunits [118, 119]. SP-D is usually composed of four subunits in a cruciform shape, however the number of subunits varies with species [120]. The more numerous the CRD domains (which is determined by the number of subunits), the higher the binding avidity to carbohydrate surfaces [118].

SP-A and SP-D bind to a wide range of microorganisms including Gram-negative bacteria, Gram-positive bacteria, yeast, fungi, and viruses [119].

**Surfactant Protein A&D Receptors**

The best characterized SP-A receptor is the SPR-210 (surfactant protein receptor 210 kDa) found on type II cells and alveolar macrophages that mediates uptake and killing of pathogens and inhibition of phospholipid secretion [121, 122]. During homeostatic conditions, SP-A and SP-D block pro-inflammatory mediators by binding to the signal-inhibitory protein α (SHIPα) receptors on macrophages through their CRDs. When the collectins bind microbes, the collagenous tails interact with calreticulin/CD91 on the cell surface thereby stimulating phagocytosis and pro-inflammatory responses [123].

**Surfactant Proteins A&D Expression**
SP-A and SP-D are primarily produced in the lower airways but are also expressed in the upper airways; including the trachea and the Eustachian tube [124]. SP-D is expressed in epithelial cells, such as the prostate and cervical mucosa [125]. Surfactant protein synthesis by alveolar type II cells begins at approximately 75% of gestation and is regulated by a wide variety of hormones, growth factors and cytokines [126]. There is complex regulation during fetal lung maturation as shown by the effect of glucocorticoids on lung development and surfactant synthesis. Glucocorticoids at low concentrations increase SP-A mRNA expression and protein, however high glucocorticoid concentrations decrease expression [127, 128]. SP-A and SP-D are expressed constitutively in the lung and expression can be increased following lung injury and epithelial cell activation.

**Surfactant Proteins A&D – Antimicrobial functions**

Antimicrobial binding is predominantly mediated by the CRD however there are reports that some organisms, such as herpes simplex virus, associate with the N-linked oligosaccharides [129]. The CRD binds to microorganisms by recognizing widely spaced, repetitive sugar arrays. The binding of pulmonary pathogens can lead to the formation of large microbial aggregates that result in the inhibition of infectivity. In addition, SP-A or SP-D can modulate the function and activity of alveolar macrophages and neutrophils thereby contributing to a more efficient clearance and killing of microorganisms [121]. SP-A is essential for host defense as shown by SP-A knockout mice that are susceptible to infection from each class of pathogen [130].
In viral infection, SP-A and SP-D can function either by direct anti-viral activity or by immune modulation [131]. The interaction of the pulmonary collectins with influenza virus is the most widely studied. SP-A interacts with influenza viral proteins through an N-linked oligosaccharide on the CRD rather than binding the carbohydrate binding site [132]. SP-D binds influenza virus through interactions of the viral haemagglutinin protein and neuraminidase envelope glycoprotein with the CRD [133]. In RSV infection, SP-A binds to the fusion glycoprotein in a calcium-dependent manner while SP-D binds the G protein to decrease viral infectivity [134-136]. Numerous pulmonary conditions are associated with an acquired deficiency of SP-D including, chronic smoking, chronic obstructive lung disease and cystic fibrosis [137, 138]. Individuals with these conditions are predisposed to viral respiratory diseases which worsen their condition.

**Surfactant Proteins A&D – Immune functions**

SP-A and SP-D modulate pulmonary inflammatory responses and primarily dampen inflammation during normal pulmonary homeostasis. Pro-inflammatory or anti-inflammatory signals stimulated by collectins depend on the cellular receptor that is activated, in addition to whether the interaction is with a host cell or a pathogen [123]. Regulation of inflammatory mediators is important to minimize tissue damage and maintaining adequate gaseous exchange. SP-A modulates the production of inflammatory cytokines produced by alveolar macrophages and thereby may also mediate the transition from innate to adaptive immunity [139]. SP-A reduces TNF-α production *in vitro* by LPS stimulated macrophages [140]. However, other studies show that SP-A
enhances TNF-α production in vitro [141], showing differential modulation depending on the environmental conditions. The collectins can also modulate the production of reactive oxygen and nitrogen species by alveolar macrophages depending on the activation state of the cell and the pathogen present [121]. SP-A and SP-D can stimulate directional actin polymerization and chemotaxis of alveolar macrophages to increase uptake of organisms. SP-A enhances LPS-mediated nitric oxide formation in alveolar macrophages that have been activated by IFN-γ but inhibit LPS-mediated production of nitric oxide in resting alveolar macrophages isolated from pathogen free rats [139]. SP-A and SP-D also function in the clearance of apoptotic cellular debris, which is important in limiting inflammation induced by lung injury [142]. Following uptake of apoptotic cells, macrophages release anti-inflammatory mediators such as IL-10, transforming growth factor β and prostaglandin E₂ [143]. Additional immune functions have been attributed to SP-A, such as regulation of dendritic cell function [144, 145] and inhibition of T cell proliferation by its collagen-like domain [146].

**Surfactant Proteins A&D in RSV Disease**

Only recently has the importance of SP-A and SP-D become evident in viral infections. In humans there are two SP-A genes (SP-A1 and 2) with multiple haplotypes. Certain haplotypes have been associated with increased risk of RSV infection, while other haplotypes are associated with protection from severe disease [147]. The importance of SP-A in RSV clearance was shown in an SP-A knockout mouse model. SP-A knockout animals inoculated with RSV showed decreased viral clearance,
increased cytokine levels and decreased superoxide and hydrogen peroxide generation by macrophages [148].

Interestingly bronchoalveolar lavage fluid from human infants with severe RSV infections reveal decreased levels of SP-A protein [149]. A recent *in vitro* study showed that although there is marked increase in SP-A gene expression without a resultant increase in protein production [150]. This study suggests that RSV may have a role in altering SP-A homeostasis and thereby innate defense mechanisms. Human infants with polymorphisms in the SP-A or SP-D genes have increased RSV severity [151, 152].

**Summary**

Surfactant proteins are important in pulmonary homeostasis and defense. SP-A and SP-D have important anti-viral actions and serve to minimize pulmonary damage during infection. Alteration in collectin expression by RSV infection or pulmonary immaturity may hinder an important component of the pulmonary innate immune response and lead to increased infection or more severe disease.

**Animal models**

There are numerous animal models used to study RSV disease. The cotton rat and mouse are the most commonly used. Many rodent models do not produce robust RSV disease. The cotton rat is an good small rodent model of RSV disease as infected animals develop both upper and lower respiratory infection with peak replication of virus at day 4 with clearance by day 7 [153]. Pulmonary lesions in the cotton rat include peribronchitis and perivasculitis with an interstitial pneumonitis. However, the infection does not
induce significant clinical signs [154]. Mouse models are frequently used due to availability of gene knock-out specific mice as well as reagent availability. BALB/c mice require a high dose of virus to become infected and develop mild inflammation, mostly surrounding blood vessels and not centered on airways [23]. The pulmonary pathology in mice does not exactly replicate that of humans, as inflammation is not centered on the airways. However, using different strains of RSV may enable better comparison. For example, BALB/c mice infected with RSV line 19 strain but not with strain A2 demonstrate increased viral load, increased IL-13, and increased bronchiolar mucus expression [155]. Increased airway mucus is a finding in human RSV infection and contributes to airway obstruction. Infant ferrets are susceptible to upper and lower respiratory infection by RSV and show age dependent susceptibility, similar to humans [156]. A rhesus monkey model developed mild clinical disease, however symptoms of more severe disease such as wheezing or dyspnea were not observed [157]. The costs associated with obtaining and housing primates hinders their frequent use in RSV studies. Other animal models include the guinea pig, hamster, chinchilla, squirrel monkeys, owl monkeys, marmosets and mink [158].

**Lamb model**

The lamb model represents an excellent model of human RSV disease. Lambs are susceptible to both human and bovine strains of RSV and develop similar pulmonary lesions to those of human infection, including bronchiolitis, airway plugging and alveolar consolidation [107, 159, 160]. Clinical disease in lambs includes fever, cough and occasional wheezing. In a study using bovine RSV, preterm lambs exhibited more severe
disease than term lambs, thereby representing an important population that is at increased risk in human medicine [161]. Importantly, pulmonary development in lambs is similar to humans. Alveologenesis begins prenatally in lambs as it does in humans. In contrast, alveologenesis occurs post-natally in rodents. The respiratory epithelia of airways, distal bronchioles and alveoli of sheep are similar to those of human lung, whereas rodents have a large number of Clara cells (50-60%) [162, 163]. Sheep also have submucosal glands which produce important innate immune products, whereas rodents do not. Finally, sheep have similar airway structure and function as humans with similar airflow, resistance and compliance [164]. There are numerous ovine pulmonary models of human disease including asthma [164], pulmonary hypertension[165], smoke inhalation [166], etc, that take advantage of the pulmonary similarity.

**Pulmonary Vascular Endothelial Growth Factor**

**Overview**

Vascular endothelial growth factor (VEGF) is a well known proangiogenic factor that also plays an important role in embryogenesis [167-169], skeletal growth [170, 171] and reproductive functions [172-174]. Mice lacking one or both VEGF-A alleles die during gestation, demonstrating that VEGF expression is critical to embryologic development [168, 175]. In addition to VEGF’s important roles in physiologic angiogenesis, VEGF has a role in pathologic angiogenesis including wound healing [176, 177], tumor vascularization [167, 178], and neovascularization associated with numerous ocular diseases [179, 180]. VEGF activates endothelial nitric oxide synthase which
mediates the permeability effects of VEGF [181]. The permeability activity of VEGF plays an important role in inflammation [182]. Numerous nonendothelial cells express VEGF including bone marrow progenitor cells [183], type II alveolar epithelial cells [184], hepatocytes, kidney podocytes, cardiac and skeletal muscle [185], lymphocytes [186], dendritic cells [187], and others. The focus here will be on VEGF expression in the lung and VEGF’s role in physiologic and pathologic processes within the lung. The lung has one of the highest VEGF mRNA expression levels of all the body tissues [188].

The VEGF gene family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor. VEGF-A is usually referred to as VEGF and is organized as eight axons separated by seven introns. Alternative exon splicing results in four predominant angiogenic isoforms; VEGF$_{121}$, VEGF$_{165}$, VEGF$_{189}$, and VEGF$_{206}$ with VEGF$_{165}$ being the predominant isoform [189, 190]. VEGF$_{165}$ is a heparin-binding homodimeric glycoprotein of 45 kDa [191]. VEGF$_{189}$ and VEGF$_{206}$ bind heparin with high affinity but VEGF$_{121}$ does not bind heparin due to its acidic nature. VEGF$_{121}$ is a freely diffusible protein while VEGF$_{189}$ and VEGF$_{206}$ are sequestered within the extracellular matrix (ECM) [192]. VEGF$_{165}$ has intermediate properties because it is both secreted and bound to the cell surface and extracellular matrix [193]. Extracellular matrix isoforms can be displaced and become active by heparin or heparinase which prevent them from binding to heparin-like moieties [192]. Plasmin can cleave ECM isoforms to allow diffusion, thereby serving a regulatory role during physiological and pathological angiogenic processes [194].
VEGF regulation at the mRNA level is complex with the formation of various isoforms in addition to anti-angiogenic isoforms. Anti-angiogenic isoforms result from alternative distal splice site selection in exon 8, which encodes an alternate COOH-terminal sequence. The anti-angiogenic protein has the same length as the pro-angiogenic isoform but a different COOH-terminal amino acid sequence and function [195]. Anti-angiogenic isoforms are listed with the letter “b” following the amino acid number, for example VEGF_{165b}. The COOH terminus of VEGF determines the proteins mitogenic potency and therefore a different terminus would relate to varied function, which are thought to regulate the angiogenic functions [196]. In vivo studies show that VEGF_{165b} significantly inhibit VEGF_{165} mediated activation of VEGF Receptor-2 (VEGFR-2) inhibiting VEGF mediated vasculogenesis [195]. A recent study examined the effect of VEGF_{121b} administration in a colon cancer allograft model system. There was marked reduction of tumor size and microvascular density in animals treated with VEGF_{121b}. Therefore anti-angiogenic properties are not attributed to just VEGF_{165b} but appear to be a common property of all isoforms with the altered terminal sequence [197].

VEGF binds two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). Both receptors have seven immunoglobulin-like domains in the extracellular domain and a single transmembrane region [198-200]. An alternatively spliced form of VEGFR-1, a soluble form, has been shown to be an inhibitor of VEGF activity [201]. VEGFR-1 is essential in embryogenesis as evidenced by Flt-1^{−/−} mice which die in utero [202]. Receptor-1 knockout mice have endothelial cells that fail to organize into vascular channels with evidence of excessive disorganization [203]. VEGFR-2 is the major
mediator of the mitogenic, angiogenic and permeability effects of VEGF. VEGFR-2 null mice fail to develop blood islands and lack organized blood vessels resulting in \textit{in utero} death [204]. VEGF also interacts with a family of co-receptors called neuropilins (NP). NP1 enhances the binding of VEGF$_{165}$ by presenting it to VEGFR-2 in a way that enhances the effectiveness of the signal transduction pathway [205].

**VEGF Regulation**

VEGF is regulated by numerous factors including hypoxia, growth factors, cytokines and other extracellular molecules [206]. Hypoxia is the most well known regulator of VEGF transcription. Under normoxic conditions, the alpha subunit of hypoxia inducing factor-1 (HIF-1) is targeted for prolyl hydroxylation which then leads to ubiquitination and degradation of HIF-1. However, under hypoxic conditions prolyl-hydroxylase is inhibited, enabling HIF-1 to enter the nucleus and binds to the hypoxia responsive elements in the promoters of hypoxia-responsive genes, including the VEGF promoter [207, 208]. VEGF is also induced by other growth factors and cytokines including IL-1, IL-6, platelet derived growth factor, TNF-\(\alpha\), TGF-\(\beta\) and fibroblast growth factor-4 [209-211].

**VEGF in Pulmonary Development**

VEGF has a complex role in lung development as VEGF is necessary for normal development of endothelial and epithelial components and plays a part in directing correct structural formation [212]. During embryogenesis, VEGF is localized within epithelial cells and within the subepithelial matrix. At the time of branching, VEGF is
concentrated at the terminal airways and stimulates neovascularization revealing that branching and vessel formation are interrelated [213]. The location of expression in addition to the concentration of protein, is crucial to development or maldevelopment. Embryonal pulmonary overexpression of VEGF results in dysmorphogenesis [214] whereas a decrease in lung VEGF results in loss of septal formation and emphysema [169].

**VEGF’s Pulmonary Functions**

VEGF has varied functions in the lung including regulation of surfactant protein synthesis, epithelial cell homeostasis and epithelial cell proliferation. VEGF acts on type II pneumocytes via the VEGFR-2 to stimulate surfactant protein production resulting in lung maturation and protection [215, 216]. Interestingly, high doses of dexamethasone, often given to neonates to increase lung maturation and surfactant production, suppress VEGF levels and VEGFR-2 expression [217, 218]. VEGF can stimulate growth of airway epithelial cells *in vitro* and may be important in epithelial cell repair [219, 220]. VEGF also serves as an anti-apoptotic factor for epithelial cells. Rats chronically exposed to a VEGF receptor blocker (SU5416) had increased alveolar septal cell apoptosis resulting in pulmonary emphysema [221]. A recent study in sheep demonstrated enhancing effects and alteration of the immune response [222]. In this study, administration of pulmonary VEGF induced the expression of IL-6, IL-8, TNFα, RANTES, MCP-1 and IFNγ by 32 hours following administration.

**VEGF in pulmonary disease**
The role of VEGF in pulmonary disease is varied and often complex. Elevated or reduced levels of VEGF have been measured in numerous respiratory disorders including pulmonary hypertension [223], acute lung injury [224, 225], COPD [226] and RSV disease [227] in addition to many others. In a fetal sheep model of pulmonary hypertension, VEGF levels are reduced and treatment with VEGF165 reduces the severity of the pulmonary vascular remodeling [223]. Acute lung injury results in the disruption of the alveolar-capillary membrane leading to disruption of oxygen exchange resulting in interstitial and alveolar edema. Patients with acute lung injury have higher plasma VEGF levels compared to control patients. High plasma VEGF levels were associated with increased mortality [224]. However, other studies measure decreased VEGF levels in lung tissue from acute respiratory distress patients and VEGF levels are negatively correlated to apoptotic endothelial cell counts [225]. A study using transgenic mice showed VEGF production induced by IL-13, led to protection against hyperoxic acute lung injury [228]. In patients with COPD there is progressive loss of lung tissue with emphysematous change. These patients have reduced levels of VEGF mRNA and protein in lung tissue. It is unknown whether the decrease in VEGF is a cause or consequence of COPD although recent studies show the link between VEGF inhibition and emphysema [226]. Patients with RSV infection demonstrate high nasal concentrations of VEGF [227]. In vitro studies using A549 cells exhibit elaboration of VEGF by RSV infected cells, in contrast to rhinovirus which does not stimulate VEGF production [229]. The role of VEGF is largely unknown in many pulmonary disease but
altered levels appear to have an effect of function. The next section will discuss VEGF as a therapeutic for pulmonary diseases.

**VEGF as a therapeutic compound**

Given VEGF’s role in surfactant homeostasis and epithelial repair, the utilization of VEGF treatment has been investigated in several pulmonary disorders including respiratory distress syndrome (RDS), hyperoxic injury and pulmonary hypertension. A study of respiratory distress in premature mice showed that intra-amniotic and intratracheal administration of VEGF increased surfactant protein synthesis, improved lung function and protected preterm mice against RDS without adverse side effects [216]. In a study of neonatal hyperoxia, treatment with VEGF increased pulmonary edema early but improved lung structure during recovery, indicating a role in reparative processes [230]. VEGF treatment in a sheep model of pulmonary hypertension reduced the severity of pulmonary vascular remodeling and preserved vasodilation, thereby reducing hypertension [223]. The side effects of VEGF treatment in the RDS, hyperoxic injury and pulmonary hypertension model were none to minimal [216, 223, 230], however there are reports of severe side effects with higher concentrations of VEGF treatment. Transgenic neonatal mice overexpressing VEGF have pulmonary hemorrhage, hemosiderosis and airspace enlargement [231]. However, VEGF levels were much higher than physiologic levels in this study. Numerous studies indicate that lower doses of VEGF are required to improve endothelial and epithelial cell functions with far fewer side effects [232, 233].
Summary

RSV animal models are vital to further understand RSV disease pathogenesis and to investigate therapeutic development. The ovine model is ideal in that pulmonary development and structure are similar to that of human lung and RSV induced pulmonary pathology is similar to human RSV disease. The pulmonary innate immune response is integral in protecting infants from severe RSV disease. Treatment with vascular endothelial growth factor prior to RSV infection may act to enhance the innate immune response, provide epithelial protection, and promote epithelial repair processes.

The specific goals of this dissertation were to:

1. describe and characterize the pulmonary pathology, antigen localization and epithelial innate responses in an ovine model of respiratory syncytial virus infection using a human RSV strain, strain A2.

2. determine the effect of pulmonary administration of human recombinant VEGF prior to RSV infection on viral load, pulmonary pathology and epithelial innate immune responses in the characterized RSV model.

3. determine the effect of ontogeny on the expression and localization of endogenous VEGF and VEGF receptors in the ovine lung.

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CHAPTER 2. HUMAN RESPIRATORY SYNCYTIAL VIRUS A2 STRAIN REPLICATES AND INDUCES INNATE IMMUNE RESPONSES BY RESPIRATORY EPITHELIA OF NEONATAL LAMBS

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Alicia Olivier, Jack Gallup, Marcia M.M.A. de Macedo,

Steven M. Varga, Mark Ackermann

Summary

Human respiratory syncytial virus (RSV) is a pneumovirus that causes significant respiratory disease in pre-mature and full-term infants. It was our hypothesis that a common strain of RSV, strain A2, would infect, cause pulmonary pathology, and alter respiratory epithelial innate immune responses in neonatal lambs similarly to RSV infection in human neonates. Newborn lambs between 2 and 3 days of age were inoculated intrabronchially with RSV strain A2. The lambs were sacrificed at days 3, 6, and 14 days post-inoculation. Pulmonary lesions in the 6-day post-inoculation group were typical of RSV infection including bronchiolitis with neutrophils and mild peribronchiolar interstitial pneumonia. RSV mRNA and antigen were detected by qPCR and immunohistochemistry respectively with peak mRNA levels and antigen at day 6. Expression of surfactant proteins A and D, sheep beta-defensin-1 and thyroid transcription factor 1 mRNA were also assessed by real-time qPCR. There was a significant increase in surfactant A and D mRNA expression in RSV-infected animals at
day 6 post-inoculation. There were no significant changes in sheep beta-defensin-1 and thyroid transcription factor-1 mRNA expression. This study shows that neonatal lambs can be infected with RSV strain A2 and the pulmonary pathology mimics that of RSV infection in human infants thereby making the neonatal lamb a useful animal model to study disease pathogenesis and therapeutics. RSV infection induces increased expression of surfactant proteins A and D in lambs, which may also be an important feature of infection in newborn infants.

**Introduction**

Respiratory syncytial virus (RSV) is a pneumovirus that causes severe respiratory disease in infants and recurrent upper airway tract infections in older children and adults. Approximately 75,000 to 125,000 hospitalizations in the United States are attributed to RSV induced bronchiolitis or pneumonia (Shay, et al., 1999). Populations at increased risk for severe disease or death include premature infants, older adults and individuals with respiratory, cardiac or immune compromise (Murata and Falsey, 2007, Welliver, 2003). There are many factors both host related and environmental that appear to be involved in the increased risk for premature infants (Welliver, 2003).

The innate immune system initiates the first line of defense against pathogens through phagocytosis and secretion of inflammatory and antimicrobial mediators. Epithelial and phagocytic cells produce and secrete antimicrobial peptides and surfactant proteins that complement and synergize with inflammatory and chemotactic mediators (Bals and Hiemstra, 2004). Epithelial cells are especially integral as they are infected by
the virus which activates receptors such as retinoic acid-induced gene 1 (RIG-1) which induces epithelial cell activation.

Lesions associated with human RSV infection include bronchiolitis, interstitial pneumonia and consolidation resulting in coughing and wheezing (Gilca, et al., 2006). Histologically, patients with RSV have bronchioles that are obstructed by mucous, fibrin and sloughed epithelial cells and neutrophils (Johnson, et al., 2007). Previously our laboratory has shown that pre-term lambs infected with bovine respiratory syncytial virus (bRSV) develop clinical responses (coughing, temperature, increased respiratory rates) and lesions that parallel those of human disease. We have also demonstrated a correlation between lamb age and disease susceptibility with more severe disease demonstrated in younger, pre-term lambs, which is similar to increased severity of RSV infection seen in human pre-term infants (Meyerholz, et al., 2004, Welliver, 2003).

The pulmonary development and cellular composition of the neonatal lamb lung are similar to that of human infants. Alveologenesis begins prenatally in both humans and lambs, in contrast to the postnatal alveolar development in mice and rodents (Flecknoe, et al., 2003, Langston, et al., 1984, Scheuermann, et al., 1988). In addition, epithelial cells of the airways, distal bronchioles and alveoli in lambs are similar to those of humans, in comparison mouse lung has a large population of Clara cells in bronchiolar airways (Mariassy and Plopper, 1983, Pack, et al., 1981). Sheep have been used to model a variety of human pulmonary diseases including asthma, pulmonary hypertension, cystic fibrosis, chronic obstructive pulmonary disease, congenital diaphragmatic hernia and numerous others (Abraham, 2008, Davey, et al., 2005, Scheerlinck, et al., 2008).
In this study, we hypothesized that a common strain of human RSV, strain A2, would infect neonatal lambs and cause pathology similar to human neonates. In addition to characterizing the pulmonary pathology we examined the duration of infection to determine the time of peak viral lesions and time at which clearance was obtained. We hypothesized that the virus would alter expression of respiratory epithelial innate immune genes known to have anti-RSV activity including surfactant proteins A (SP-A) and D (SP-D) and sheep beta-defensin-1 (SBD-1). Expression of thyroid transcription factor-1 (TTF-1), a key SP-A transcription factor, was also measured.

Materials and methods

Experimental Design

Animal use and experimental procedures were approved by Iowa State University’s Animal Care and Use Committee. Neonatal lambs (2-3 days of age) were randomly assigned to two groups, a control group (n=10) or RSV inoculated group (n=18). Lambs were anesthetized with an intramuscular injection of xylazine (0.1 mg/kg), placed in right lateral recumbency and inoculated with human respiratory syncytial virus, strain A2 (5 ml of 2x10^7 pfu/ml RSV) via fiberoptic bronchoscope in the right terminal bronchus followed by a sterile saline flush (10 ml). Control animals were inoculated with cell growth media (5 ml) without the virus followed by a sterile saline flush (10 ml). RSV (A2 strain) was a gift from Barney S. Graham (National Institutes of Health; NIH, Bethesda, MD) and was grown in HEp-2 cells (American Type Culture Collection; ATCC, Manassas, VA). Lambs were given daily antibiotics (ceftiofur, 2.2 mg/kg,
intramuscular) to prevent bacterial complications. Lambs were monitored for clinical signs of respiratory disease (coughing and wheezing) and daily temperatures were measured. Animals were sacrificed by sodium pentobarbital on days 3 (control n=3, RSV n=6), 6 (control n=4, RSV n=6) and 14 post-inoculation (control n=3, RSV n=6).

**Tissue**

The thorax was opened and the lungs were examined for gross lesions. The lungs were then removed for tissue collection. In all animals, tissue samples were taken at the same location of the right cranial, middle and caudal lobes and left cranial lung lobe. Samples were placed in cassettes and then in 10% neutral-buffered formalin for histological and immunohistochemical analysis. Additional samples were taken from these sites and snap-frozen on dry ice for real-time quantitative PCR (Meyerholz, et al., 2004).

**One-step real-time qPCR**

Total RNA was isolated from whole lung tissue (affected areas as determined grossly or by immunohistochemistry for RSV antigen) via Trizol according to manufacturer’s guidelines (Invitrogen). RNA samples were assessed by spectrophotometry and DNase treated (Ambion, TURBO DNase). Real-time quantitative PCR (qPCR) was carried out as a fluorogenic one-step process in a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using previously described methods. Primer and probe sequences used in our laboratory have been previously described (Hu, et al., 2003, Kawashima, et al., 2006)(Table 1). All samples were run in duplicate and each target gene amplification
was converted to a relative quantity and normalized to the geometric mean of two housekeeping genes, hRibo18S and ovRPS15 (Gallup and Ackermann, 2006).

**Histologic Examination**

The lung histologic lesion score was determined by the percentage of parenchymal consolidation of each section of lung and immunohistochemical antigen staining for RSV. The scoring scale for the alveolar consolidation was; 0, no consolidation; 1, <30%; 2, 30-60%; 3, >60%. The scoring scale for the RSV antigen staining was; 0, no staining; 1, 1-5 positive cells; 2, 6-10 positive cells; 3, >10 positive cells. All evaluations were made based on the average of 10, 20X fields per section and 4 sections per lung per animal. Group averages were calculated for the RSV antigen score and the alveolar consolidation score.

**Immunohistochemistry**

Immunohistochemistry for RSV antigen was performed on paraffin-embedded tissue as described previously (Meyerholz, et al., 2004). Briefly, sections were cut at 5 µm thickness onto positively charged slides. Following routine deparaffinization, sections were treated with Pronase E (Protease Type XIV from *Streptomyces griseus*, Sigma) for 12 minutes at 37°C. Nonspecific binding was blocked by incubation in 20% normal swine serum. The sections were incubated with polyclonal goat anti-RSV antibody (BioDesign/Meridian) overnight at a concentration of 1:50 with 5% normal swine serum. The slides were rinsed and then incubated with biotinylated rabbit anti-goat secondary antibody (KPL) at a concentration of 1:300 in 5% normal sheep serum. A peroxidase
block with 3% peroxide was followed by incubation with peroxidase-conjugated streptavidin (BioGenex) for 45 minutes. After two PBS washes, the color was developed with Nova Red. The slides were then counterstained with Harris’ hematoxylin, dehydrated and cover-slipped.

**Statistical analysis**

Data are expressed as means ± SEM. For the temperature data, the group means on each day were analyzed for significance (RSV infected versus control) using unpaired student t-tests. The histologic lesion score data was analyzed using the Wilcoxon-Mann-Whitney test. The RT-qPCR data was LOG-transformed to stabilize variances across means and bring data to a normal distribution. A two-way ANOVA (unbalanced design) was used to test for significant effects of treatments (control versus RSV infected), groups (3, 6, and 14 days post-inoculation time-points), and their interaction. A two-sample t-test was used to compare target genes expression at post-inoculation time-points. The Pearson product-moment correlation coefficient was used to evaluate relationships between hRSV levels and expression of various target genes.

**Results**

**Clinical and post-mortem findings**

RSV-infected lambs had significantly higher temperatures than control animals at days 1-6 post-inoculation (Figure 1, \( P < 0.05 \)). Clinically, 3 of the 6 lambs in the 6 day post-inoculation group developed a slight to moderate cough on days 4 and 5 post-inoculation. 2 of the 6 lambs in the 14 day post-inoculation group developed a moderate
cough on day 5 post-inoculation with resolution of the cough at day 10. At post-mortem examination gross lesions were characterized by multifocal to locally extensive reddened areas of pulmonary consolidation which varied in severity from moderate to severe. In the 6-day post-inoculation group, 5 of the 6 infected animals had gross lesions. No gross lesions were present in the 3 and 14-day post-inoculation group.

**Histology and immunohistochemistry**

Histologically, RSV induced changes were characterized by mild to moderate suppurative bronchiolitis with sloughed epithelial cells, cellular debris and neutrophils in the lumen of the medium to small airways at days 3 and 6. Also present was a mild to moderate peribronchiolar mononuclear interstitial pneumonia containing lymphocytes and plasma cells and locally extensive areas of alveolar consolidation (Figure 2 A and B). In the 3-day post-inoculation group, all six lambs inoculated with RSV had histological changes consistent with RSV infection. In the 6-day post-inoculation group, 5 of the 6 RSV inoculated lambs had histological changes associated with RSV virus. In the 14-day post-inoculation group, 3 of the 6 RSV inoculated lambs had minimal changes characterized by multifocal small areas of alveolar consolidation.

Immunoreactivity for RSV antigen was present within the airway, bronchiolar epithelium and the syneytial cells in consolidated areas in the 3 and 6-day post-inoculation groups (Figure 2 C and D). Immunoreactivity to RSV antigen was not present in the 14-day post-inoculation group. A histologic score was calculated for RSV immunoreactivity and alveolar consolidation for each group (Figure 3). Animals in the 6
day RSV post-inoculation group had significantly higher scores for RSV immunoreactivity and alveolar consolidation as compared to the 3 day RSV post-inoculation group ($P < 0.05$). There was a significant decrease in both lesion scores in the 14 day RSV post-inoculation group ($P < 0.05$).

**Epithelial innate immune gene expression**

Expression of RSV, SP-A, SP-D, SBD-1 and TTF-1 were measured by qPCR. The analysis showed significant increase in RSV mRNA from day 3 to day 6 post-inoculation ($P < 0.01$) and a significant decrease from day 6 to 14 post-inoculation ($P < 0.01$) (Figure 4). The analysis showed statistically significant differences in SP-A and SP-D levels between infected and control groups. There was a significant ($P < 0.05$) increase in expression of SP-A between RSV-infected and control animals in all three groups. However, there was no significant difference in expression of SP-A in the RSV-infected lambs between different post-inoculation time-points (Figure 5). There was a significant increase in expression of SP-D between 3-day and 6-day post-inoculation animals and a significant decrease between 6-day and 14 days post-inoculated animals (Figure 6, $P < 0.03$). There was a significant difference between control and infected animals in the 6-day post-inoculation group ($P < 0.03$). In addition there was a correlation between RSV levels and SP-D expression in infected animals. There were no significant differences in SBD-1 and TTF-1 expression between the infected and non-infected groups.
Discussion

Neonatal lambs infected with human respiratory syncytial virus (RSV), strain A2 develop lesions consistent with those seen in human infant RSV infection (Johnson, et al., 2007, Welliver, et al., 2007). Histologically, bronchiolitis was characterized by infiltration of neutrophils and macrophages into the bronchiolar lumen admixed with degenerate epithelial cells and cellular debris. In addition, there were multifocal to locally extensive areas of alveolar consolidation with numerous syncytial cells and infiltrates of lymphocytes and plasma cells at days 6 and 14. Pulmonary lesions are also similar to those that occur experimentally in lambs with bRSV and in natural spontaneous lesions of bRSV-infected cattle (Lehmkuhl and Cutlip, 1979). Pulmonary pathology was most severe at 6 days post-inoculation at which time animals exhibited cough and high temperatures. By 14 days post-inoculation, lambs had almost complete resolution given the lack of gross lesions, histologic changes and immunoreactivity for RSV antigen at this time point. The infection and disease progression correlates to a previous study of experimental RSV infection in lambs in which infected lambs showed clinical signs associated with RSV infection, however, in that study tissue collection was carried out four weeks following inoculation, therefore no histological changes were present (Lapin, et al., 1993).

The perinatal lamb has several key features consistent with human infant RSV infection and pulmonary development that make it a very good animal model. As shown here, lambs develop histological lesions similar to human infection. Robust RSV pathology is lacking in many rodent models of human RSV infection thereby making the
ovine model more attractive (Kong, et al., 2005). These lesions represent a moderate human infection such that resolution is possible, which commonly occurs in infants. The severity of pneumonia in this model can likely be enhanced by increasing the viral density and volume of viral inoculum.

Associated with gross lesions were significant increases in expression of surfactant proteins A and D (Figures 5 and 6). The increase in SP-A expression is similar to studies with lambs inoculated with bRSV and indicates a cellular response to the virus (Kawashima, et al., 2006). Both SP-A and SP-D have anti-RSV activity which include viral opsonization and activation of macrophages, which are thought to play a critical role in RSV clearance (Hickling, et al., 2004, Sano and Kuroki, 2005). In severe human RSV infection, SP-A protein levels are decreased (Kerr and Paton, 1999). Our previous work has shown that paramyxoviral infection in sheep increases SP-A gene expression but does not significantly increase SP-A protein levels (Grubor, et al., 2004). Human individuals with deleterious polymorphisms in the SP-A gene show increased severity of RSV infection – indicating the importance of SP-A in viral clearance and demonstrating the importance of this protein clinically (Lahti, et al., 2002). Moreover, SP-D enhances RSV macrophage uptake and plays a role in modulating the immune response. Mice deficient in SP-D exhibit impaired RSV clearance and an increased neutrophilic response and human SP-D polymorphisms are associated with either increased severity of RSV or protection against severe infection (Lahti, et al., 2002, LeVine, et al., 2004, Pastva, et al., 2007).
Beta defensin expression in the lung is developmentally regulated in both humans and sheep (Meyerholz, et al., 2006, Starner, et al., 2003). Beta defensins are produced by pulmonary epithelial cells and leukocytes and have antimicrobial properties (Hickling, et al., 2004, Schutte and McCray, 2002). In this study, SBD-1 expression was not significantly altered by RSV infection. In humans, HBD-1 is constitutively produced in the lung and is not inducible by pro-inflammatory mediators (Starner, et al., 2005). This may be similar in the ovine and explain the lack of up-regulation of SBD-1, however, in previous studies, SBD-1 was up-regulated with parainfluenza virus (PIV-3) indicating that under certain conditions this gene may be inducible (Grubor, et al., 2004).

Thyroid transcription factor-1 is a nuclear transcription factor that is most prevalent in the type II epithelial cells in the alveolus in the perinatal lung. TTF-1 binds to regulatory promoter elements of surfactant protein A in addition to other surfactant proteins (DeFelice, et al., 2003). TTF-1 mRNA levels did not increase during RSV infection despite the increases in SP-A mRNA levels. This may indicate that other regulatory factors are involved or that the duration in which there is up-regulation of the transcription factor is at an earlier time-point prior to our sample collection (e.g. day 3). The regulation of surfactant protein expression is complex involving multiprotein signaling complexes. TTF-1 interacts with many other transcription factors and co-factors such as FOXa2, GATA-6, AP-1, C/EBPa, NFATc3 and retinoic acid receptors (Besnard, et al., 2007, Dave, et al., 2004). It is also possible that SP-A up-regulation could be downstream to RIG-1 activation.
We have previously shown that pre-term lambs infected with bRSV have more severe RSV lesions than full-term lambs (Meyerholz, et al., 2004). We expect that infection of pre-term lambs with this human strain of RSV would similarly have increased lesion severity similar to human premature infant disease.

This study shows that the pulmonary pathology of RSV in neonatal lambs is similar to a moderate human infant RSV infection. This is the first time that the pulmonary epithelial innate immune response has been characterized in the neonatal lamb model of human RSV. In this study, RSV infection up-regulated surfactant proteins A and D expression but did not alter the expression of sheep beta-defensin-1 or thyroid transcription factor. Future work in our laboratory using this model will elucidate other key roles of epithelial cells in RSV infection and allow a useful model for therapeutic trials.

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**References**


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6FAM or VIC, 5’ Fluorescent reporter dye; TAMRA, Fluorescent quencher dye; MGBNFQ, Minor groove binding non-fluorescent quencher dye.

Table 1. Primers and probe sequences for ovine gene expression assessed by real-time qPCR.
Figures:

![Graph showing average body temperatures by control and RSV infected lambs.](image)

**Figure 1:** Average body temperatures by control and RSV infected lambs. RSV infected lambs had significant increases in body temperature (*$P < 0.05$). Values are expressed as means ± SD.
Figure 2: **A.** Lung from a control lamb not infected with RSV that contains a bronchiole surrounded by non-collapsed alveoli with a central lumen. **B.** Lung from a lamb 6 days post-inoculation with RSV. Within the lumen of the bronchiole (outlined) are sloughed epithelial cells mixed with degenerate neutrophils (*). The alveoli surrounding the bronchiole are collapsed with accumulation of degenerate neutrophils and areas of necrosis (arrow). **C and D.** Immunohistochemistry for RSV antigen on lung tissue from a lamb 6 days post-inoculation with RSV, in which there is immunoreactivity within the bronchi, epithelial cells lining the bronchi, and the syncytial cells in areas of alveolar consolidation. Bars = 25 µm.
Figure 3: Histologic lesion score based on RSV antigen staining and alveolar consolidation of RSV infected animals at 3, 6, and 14 days post-inoculation. RSV antigen staining and alveolar consolidation scores were highest at day 6 post-inoculation. Values are expressed as means ± SD. +P < 0.05 vs other time points.
Figure 4: Comparison of mRNA levels of RSV between groups. There was a significant increase in the expression of viral mRNA between RSV infected vs control animals at 3 and 6 days post-inoculation (*$P < 0.01$). There was a significant increase in expression of RSV between RSV infected animals at 6 days post-inoculation vs other time points ($^{+}P < 0.01$). Control animals lack RSV mRNA. Values are expressed as means ± SD.
Figure 5: Comparison of mRNA levels of SP-A between groups. There was a significant increase in expression of SP-A between RSV infected vs control animals (*$P < 0.05$) at all post-inoculation time points. There was no significant difference in expression of SP-A in the RSV-infected lambs between different post-inoculation time-points. Values are expressed as means ± SD.
Figure 6: Comparison of mRNA levels of SP-D between groups. There was a significant increase in expression of SP-D between RSV infected vs control animals at the 6-day post-inoculation time point (*P < 0.03). There was a significant increase in expression of SP-D between RSV infected animals at 6 days post-inoculation compared to other time points (†P < 0.03). There was a correlation between RSV levels and SP-D expression in infected animals (r=0.60, P < 0.008). Values are expressed as means ± SD.
CHAPTER 3. EXOGENOUS ADMINISTRATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR PRIOR TO HUMAN RESPIRATORY SYNCYTIAL VIRUS A2 INFECTION REDUCES PULMONONARY PATHOLOGY AND ALTERS EPITHELIAL INNATE IMMUNE RESPONSES

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Alicia K. Olivier, Jack M. Gallup, Albert van Geelen, Mark R. Ackermann

Abstract

Human respiratory syncytial virus (RSV) affects thousands of children every year. Vascular endothelial growth factor (VEGF) is a regulator of vasculogenesis, pulmonary maturation and immunity. In order to test the extent to which VEGF may alter RSV infection, four groups of lambs received either human recombinant VEGF (rhVEGF) or PBS pretreatment followed by inoculation with human RSV, strain A2 or sterile media. Lambs in each group were sacrificed at 2, 4 and 6 days post-infection. Expression of surfactant protein-A (SP-A), surfactant protein-D (SP-D), sheep beta-defensin-1 (SBD-1), tumor necrosis factor α (TNFα), IL-6, IL-8, interferon β and endogenous VEGF were measured to determine effect of rhVEGF pretreatment. RSV lambs pretreated with rhVEGF had reduced viral mRNA and decreased pulmonary pathology at day 6. Pretreatment with rhVEGF increased mRNA expression of SP-A, SBD-1, and TNFα with alteration of expression in RSV lambs. Endogenous VEGF mRNA levels were increased at day 2 regardless of pretreatment. Pretreatment with rhVEGF increased pulmonary cellular proliferation in RSV lambs at day 4 post-infection. Overall, these results suggest that pretreatment with rhVEGF protein may
have therapeutic potential to decrease RSV viral load, decrease pulmonary lesion severity and alter both epithelial innate immune responses and epithelial cell proliferation.

INTRODUCTION

Respiratory syncytial virus (RSV) is a pneumovirus in the paramyxoviridae family. RSV is the most significant cause of acute respiratory tract infection in infants [1]. Thousands of children are affected by RSV every year with more severe infection in premature and respiratory compromised infants [2]. There are few effective treatments for RSV, however prophylactic administration of an anti-RSV monoclonal antibody has been effective in decreasing disease in high risk infants and children [3]. Until an effective vaccine is developed there is still a great need to find and develop other therapeutic targets to decrease virus induced pathology.

Vascular endothelial growth factor (VEGF) is a potent endothelial cell growth factor, mitogen, and survival factor [4] that has well-known vascular permeability functions important in inflammation and wound healing [5, 6]. VEGF has numerous non-endothelial functions and is highly expressed in tissues with specialized epithelial barriers such as the lung and kidney [7]. In the lung, VEGF is expressed by alveolar type II cells, bronchiolar epithelial cells [8] and alveolar macrophages [9, 10]. Given the high pulmonary expression there is a vast array of research examining altered levels of VEGF in pulmonary homeostasis and disease.

VEGF-A is a member of the vascular endothelial growth factor family which also includes VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor. The gene
encoding VEGF-A is comprised of eight exons that are alternatively spliced to yield multiple protein isoforms with varying activities [6, 10]. The isoforms include VEGF\textsubscript{121}, VEGF\textsubscript{145}, VEGF\textsubscript{165}, VEGF\textsubscript{189} and VEGF\textsubscript{206}. VEGF\textsubscript{165} is the predominant isoform and the most potent [11]. VEGF\textsubscript{121} is freely diffusible while VEGF\textsubscript{189} and VEGF\textsubscript{206} are bound within the extracellular matrix. VEGF\textsubscript{165} is both diffusible and sequestered to the cell surface and extracellular matrix via its heparin binding moieties.

During pulmonary development adequate levels of VEGF are critical for the proper formation of pulmonary capillaries. Inactivation of the VEGF-A gene in lung epithelia of mice embryos results in almost complete absence of pulmonary capillaries with abnormal alveolar septae formation [12]. Treatment of rats with a VEGF receptor blocker (SU5416) leads to alveolar apoptosis and subsequent emphysema, demonstrating that VEGF is necessary for the maintenance of alveolar structure [13]. There is controversy and conflicting data concerning the role of altered VEGF expression in acute and chronic pulmonary disease. Previous in vitro studies show that RSV induces increased VEGF expression [14, 15]. This does not appear to be the case for all respiratory viruses as infection with rhinovirus does not result in an increase in VEGF expression [16].

We previously demonstrated that lambs inoculated with human RSV, strain A2 developed lesions similar to those of human neonates [17, 18] with peak infection observed at 6 days post-infection [18]. Ovine lung is a good pulmonary model as the pulmonary structure and development are similar to human lung and sheep are susceptible to human strains of RSV. In addition, the respiratory epithelia of the airways, bronchioles and alveoli in sheep are composed of similar ratios in cell types as humans, whereas mice have large
populations of Clara cells in the bronchiolar airway [19, 20]. Sheep have similar bronchiolar glands, airway capillaries and cough reflexes as humans [21].

In this study, we hypothesized that pretreatment with recombinant human (rh) VEGF prior to infection with human RSV, strain A2, would increase expression of important epithelial innate immune genes and thereby increase viral clearance and reduce pulmonary pathology. Additionally, we proposed that transcription of endogenous VEGF and VEGF receptors in the lung would be altered by RSV infection.

METHODS

Experimental Procedure

Animal use and experimental procedures were approved by Iowa State University’s Animal Care and Use Committee. Neonatal lambs (2-3 days of age) were randomly assigned to 3 main groups; 2 day (n=16), 4 day (n=16) and 6 day (n=24) based on length of infection period. Within each of these groups, animals were then assigned to treatment groups (Table 1). All lambs were pretreated with recombinant human VEGF165 (30µg/kg) (Invitrogen or GenWay Biotech, Inc.) or phosphate-buffered saline (PBS). Animals in the day 2 and 4 groups were treated via intrabronchial administration while those in the day 6 group were treated via intratracheal administration. Thirty minutes following pretreatment, animals were inoculated with human RSV virus (10 ml, 3 x 10^6 pfu/ml) or cell growth media (10 ml). RSV (A2 strain) was propagated in HEp-2 cells (American Type Culture Collection; ATCC, Manassas, VA). The inoculum was a cleared supernatant from infected cells that underwent a centrifugation step to remove cellular debris. The virus was tittered via plaque assay on
VERO cells according to standard protocols. Prior to and following inoculation, lambs were administered daily antibiotics (ceftiofur, 2.2 mg/kg, intramuscular) to prevent bacterial pulmonary complications. Lambs were monitored daily for clinical signs of respiratory disease (coughing and wheezing) and temperatures were recorded. Animals were sacrificed by sodium pentobarbital on days 2, 4 and 6 post-inoculation.

Tissue samples were taken at the same location of the right and left cranial, middle, caudal lobes and accessory lobe. Samples were placed in cassettes and then in 10% neutral-buffered formalin in preparation for histologic analysis. Additional lung samples were taken from these sites and snap-frozen on dry ice for real-time quantitative PCR.

**Immunohistochemical analysis**

Immunohistochemical staining was performed for RSV antigen (1:50, overnight incubation, goat anti-human polyclonal antibody, BioDesign/Meridian) and for proliferating cells using an antibody against the Ki67 protein (1:100, overnight incubation, mouse anti-human monoclonal antibody; Dako). Briefly, paraffin-embedded lung sections were cut at 5µm thickness onto positively charged slides. Slides were incubated at 58°C for 25 minutes, deparaffinized in xylene, rehydrated in graded ethanol washes and washed in phosphate-buffered saline (PBS). For RSV immunohistochemistry, tissue sections were treated with Pronase E (Protease Type XIV from Streptomyces griseus, Sigma) for 12 minutes at 37°C. Nonspecific binding was blocked by incubation in 20% normal swine serum. For Ki-67 immunohistochemistry, tissue sections were boiled for 15 minutes in 0.01M citric acid (pH 6.0). Following overnight incubation with the primary antibodies the slides were rinsed and then incubated with an appropriate biotinylated secondary antibody at a concentration of
A peroxidase block with 3% peroxide was followed by incubation with peroxidase-conjugated streptavidin (BioGenex) for 45 minutes. After three PBS washes, the color was developed with Nova Red. The slides were then counterstained with Harris’ hematoxylin, dehydrated and cover-slipped.

**Histology scoring**

Histologic lesion scores were determined from two lung sections per RSV infected lamb in each pre-treatment group (e.g., VEGF vs. PBS). Sections were scored by a pathologist blinded to the study groups to determine total alveolar consolidation and cellular infiltration. Alveolar consolidation was defined by reduced expansion of alveolar lumen due to alveolar septal infiltration of neutrophils, lymphocytes, plasma cells, and type II cell hypertrophy along with intralumenal accumulation of neutrophils, macrophages and small amounts of cell debris. A total of five 20X fields were examined per lung section and alveolar consolidation was scored as: 0, no consolidation; 1, 1-30% consolidation; 2, 30-50% consolidation, 3, 60-80% consolidation, 4, >80% consolidation. Values were averaged per section and converted to a percentage value.

Two lung sections from all animals were examined to determine the number of Ki-67 immunoreactive cells. A total of ten 20X fields were examined per lung section and values were averaged.

**One-Step Real-Time qPCR**

Total RNA was isolated from whole lung tissue (affected areas as determined grossly or by immunohistochemistry for RSV antigen) via Trizol according to manufacture’s
guidelines (Invitrogen, Carlsbad, CA). Following RNA isolation, the pellets were resuspended in 0.1mM EDTA, pH 6.75 and heated at 65°C for 5 min. Samples were diluted 1:50 and absorbance readings at 260 and 280nm were measured to determine RNA concentration and purity. RNA samples were DNase treated (TURBO DNase, Ambion) and diluted 1:10 with nuclease-free water and RNAseOUT (Invitrogen) for one-step real-time qPCR. Reactions were carried out using SuperScript™ III Platinum® One-Step quantitative RT-PCR System with ROX kit (Invitrogen). A test plate was run using a mixture of RNA samples serially diluted to determine the optimal dilution range for each target. Primer and probe sequences used in our laboratory have been previously described [18, 22, 23] (Table 2). The ovine VEGF primers and probe detected ovine VEGF isoforms (VEGF120, VEGF164, VEGF187). Following determination of the optimal dilution range for each target, samples and standards were run in duplicate [24]. Each target gene amplification was converted to a relative quantity using a target specific standard curve and then normalized to the reference gene, ubiquitin and OvRPS15 [25].

Statistical Analysis

Statistical analyses were performed with commercially available statistics software (GraphPad Prism, GraphPad Software). Data was analyzed for normal distribution and log transformed when necessary. Comparisons were done using one-way ANOVA followed by Tukey’s post-test analysis. Histologic lesion score data was analyzed using a Student t-test. Data are represented as means (SEM). $P < 0.05$ was considered to be significant unless otherwise noted.
RESULTS

Clinical Data

Lambs infected with RSV had increased body temperatures at days 2 and 3 post-infection regardless of pretreatment group (data not shown). Lambs in both pretreatment groups (PBS and VEGF) exhibited a moderate cough at days 4 and 5 post-infection.

Histology and Immunohistochemistry

Histologic lesions of RSV infected lambs were characterized by multifocal bronchiolitis with locally extensive alveolar consolidation. Within the lumen of bronchi and bronchioles were neutrophils and macrophages admixed with cellular debris. Alveoli surrounding affected bronchi were collapsed and infiltrated by neutrophils with fewer lymphocytes and plasma cells (Figure 1A). Syncytial cells were present multifocally within bronchiolar epithelium and within consolidated alveolar parenchyma in lung tissue from PBS and VEGF pretreated lambs. RSV antigen was detected in bronchiolar epithelial cells, syncytial cells and alveolar macrophages by immunohistochemistry (Figure 1B). Immunoreactive cells had intense, dark brown, cytoplasmic staining. At day 6 post-infection, RSV infected lambs pretreated with rhVEGF had decreased alveolar consolidation, bronchiolitis and inflammatory cell infiltration as compared to RSV infected lambs pretreated with control media (Figure 1C). There was reduced RSV antigen at day 6 post-infection in rhVEGF pretreated lambs (Figure 1D). Histologic lesion scores were calculated for each treatment group based on alveolar consolidation and inflammatory cell infiltration (Figure 2).
Real-time qPCR

Effect of rhVEGF on RSV mRNA expression

RSV mRNA levels were measured by RT-PCR to determine the effect of rhVEGF pretreatment on RSV replication. Lambs pretreated with rhVEGF had significantly higher RSV mRNA levels at day 2 post-infection than RSV infected lambs pretreated with PBS. However at day 6, lambs pretreated with rhVEGF had a marked decrease in RSV mRNA levels as compared to lambs pretreated with PBS (Figure 3).

Effect of RSV infection and rhVEGF administration on collectin and defensin gene expression

Surfactant protein and defensins serve important roles in the pulmonary innate immune response. Expression of SP-A, SP-D and SBD-1 mRNA were measured in order to determine the effect of rhVEGF pretreatment on the expression of these genes during RSV infection. rhVEGF induced the expression of SP-A in non-RSV infected lambs pretreated with VEGF at day 4 and 6, indicating increased expression was a direct result of rhVEGF administration (Figure 4). RSV infection induced expression of SP-A at day 4 in both pretreatment groups. At day 6, RSV infected lambs pretreated with rhVEGF showed a marked reduction in SP-A expression compared with lambs pretreated with PBS at the same time point and expression at day 4. There were no significant changes in SP-D expression between the groups.

Administration of rhVEGF induced significant increase of SBD-1 mRNA in uninfected lambs at day 4 and 6, indicating increased expression was a direct result of
rhVEGF (Figure 5). RSV infection induced expression of SBD-1 at days 2 and 4 post-infection in both pretreatment groups, showing that increased expression was a result of viral infection. At day 6, RSV infected lambs pretreated with rhVEGF had less SBD-1 expression than lambs pretreated with PBS and lower levels than those present at day 4.

**Effect of RSV infection and rhVEGF administration on inflammatory mediators**

RSV infection induced the expression of TNFα and IL-8 at day 2 post-infection in both pretreatment groups (Figures 6, 7). Administration of rhVEGF increased TNFα expression at day 4 (Figure 6). There was no significant alteration in expression of IL-6 and IFNβ by RSV infection or rhVEGF administration.

**Alterations in endogenous VEGF expression due to RSV infection**

Expression of VEGF and VEGF receptors-1 and -2 by lung cells was measured to determine whether RSV infection and/or the administration of rhVEGF protein altered transcription of endogenous VEGF and cellular regulation of VEGF receptors-1 and -2. VEGF mRNA was significantly increased in RSV infected lambs at day 2 in both pretreatment groups (Figure 8). However, at day 6, levels of VEGF mRNA were markedly decreased in both pretreatment groups. VEGF receptor transcription was not altered by viral infection or administration of rhVEGF (data not shown).

**Effect of RSV infection and rhVEGF administration on pulmonary cellular proliferation**

VEGF can reduce apoptosis of both endothelial cells and pulmonary epithelial cells [13, 26]. The effect of VEGF on the growth and proliferation of lung epithelial cells following injury is not well known. To test this, cellular proliferation was measured within
the lung parenchyma by immunohistochemistry for Ki-67 antigen (Figure 9). Immunoreactive cells had intense, dark brown, nuclear staining. Immunoreactive cells were present within bronchiolar epithelium and multifocally within the alveolar interstitium. At day 4, RSV infected lambs pretreated with rhVEGF had increased cellular proliferation compared to control (Figure 10). There was no significant difference in RSV infected lambs pretreated with PBS at this time point compared to PBS control. At day 6, there were significantly more Ki-67 immunoreactive cells in RSV infected lambs pretreated with PBS compared to lambs pretreated with VEGF. In fact, RSV infected lambs pretreated with rhVEGF had no increase in cellular proliferation over control at day 6.

DISCUSSION

VEGF has a vital role in pulmonary development, homeostasis and disease processes. This study demonstrates that administration of exogenous rhVEGF prior to RSV infection decreases the severity of RSV induced pulmonary lesions at day 6 post-infection. In addition, viral load at 6 days post-infection was decreased in lambs pretreated with rhVEGF. Demonstration of rhVEGF-induced protection against RSV is consistent with our previous work demonstrating pulmonary protection with a bovine strain of RSV [27]. The mechanistic basis for decreased RSV pathology is largely unknown, however we propose that rhVEGF increases innate immune gene expression by pulmonary epithelial cells and promotes epithelial cell proliferation leading to enhanced cellular repair. VEGF has numerous other pulmonary effects that may contribute to alteration of RSV disease including vascular leakage and macrophage activation. An unexpected finding was an increase in RSV mRNA at day 4 post-infection in rhVEGF pretreated lambs. The cause for the higher expression is unknown but may be related to alteration in cellular responses by rhVEGF.
There was no histologic evidence of increased cellular infiltration or pulmonary pathology that would indicate increased RSV disease at this time point.

SP-A is a collectin that plays a critical role in pulmonary innate immune responses and also modulates inflammatory responses. SP-A acts as an opsonin by binding and agglutinating pathogens and also serves to modulate inflammatory responses in the lung [28]. Exogenous pulmonary VEGF administration increases SP-A expression and expression of other surfactant proteins, resulting in pulmonary maturation and making VEGF a possible therapeutic for pulmonary immaturity [29, 30]. Other routes of VEGF administration also increase pulmonary surfactant expression. In a preterm rat study, rats given VEGF via intra-amniotic administration resulted in increased expression of SP-B and SP-D [31]. SP-A has important anti-viral functions including direct virus binding and immune modulation [32]. Human infants with polymorphisms in the SP-A gene show increased susceptibility to RSV infection [33]. Studies using SP-D knockout mice demonstrate that RSV infection results in impaired viral clearance with increased infiltration of neutrophils [34]. In this study, lambs pretreated with rhVEGF had markedly increased SP-A expression, similar to other studies using exogenous rhVEGF administration. Lambs infected with RSV alone had increased expression of SP-A, corresponding to previous studies showing that RSV induces SP-A expression [18]. Interestingly at day 6 post-infection, SP-A expression in RSV infected lambs pretreated with rhVEGF was much lower than animals pretreated with PBS and also decreased from day 4. Although rhVEGF induces expression of SP-A there appears to be modulation of this effect when lambs are infected with RSV. Reduced expression of SP-A at
day 6 in RSV infected lambs pretreated with VEGF may be secondary to the effects of the rhVEGF, which may decrease viral load and result in less viral induction of SP-A.

Beta defensins are antimicrobial peptides that have important roles in both the innate and adaptive immune responses. The antiviral activity of β-defensins include direct anti-viral activity, chemotactic activity for macrophages and immature dendritic cells, and induction of cytokine signaling by monocytes and epithelial cells [35]. RSV infection induced expression of SBD-1 at 2 and 4 days in both pretreatment groups. Pretreatment with rhVEGF in non-RSV infected lambs induced expression of SBD-1 at days 4 and 6. The induction of SBD-1 expression by VEGF has been documented in a previous study showing that VEGF modulates components of the innate immune response [27]. At day 6, SBD-1 levels in RSV infected lambs pretreated with VEGF were much lower than lambs pretreated with PBS. The lower expression level in this group is similar to the decreased expression observed with SP-A. Again, the cause for the lower expression in this group is unknown, but may be related to a decrease in viral-induced epithelial responses.

A study investigating VEGF levels in human infants with RSV infection showed up-regulation of VEGF in the nasal fluid [36]. In many human studies nasal fluid and bronchoalveolar lavage (BAL) are the only means of measurement, as tissue samples are often difficult to obtain. In this study, endogenous VEGF expression was increased at day 2 but decreased at day 6. VEGF mRNA was measured in lung homogenates, which may give an indication of the overall effect of RSV-induced inflammation on VEGF expression. VEGF is secreted both basolaterally and apically by pulmonary epithelial cells. In addition, VEGF_{189} and VEGF_{206} are bound to the extracellular matrix within the lung tissue and
cleaved to active fragments by plasmin upon extracellular matrix breakdown [4]. Nasal secretion and BAL measurements provide information as to the expression level in one location only. An example of the location differences in VEGF levels can be shown in a study using a neonatal pig lung injury model, which reported decreased VEGF mRNA expression and protein levels in lung tissue with increased protein levels in the BAL [37]. This poses the question as to the significance of altered expression at various locations and time points in infection.

Many pulmonary disease processes can result in or be caused by alterations in VEGF expression. Decreased pulmonary VEGF expression is present in newborns with bronchopulmonary dysplasia and in acute respiratory distress syndrome [38, 39]. In one murine model of LPS-induced lung, VEGF mRNA and protein expression were increased following injury with evidence of pulmonary edema [40]. However, in a different model of acute lung injury induced by *Pseudomonas aeruginosa*, VEGF mRNA and protein were decreased [41]. In the injury model induced by *Pseudomonas*, direct injury to the epithelium may have resulted in decreased expression, although decreased levels may be an epithelial response to injury. The fact that RSV infected lambs in both pretreatment groups showed similar VEGF mRNA expression may indicate a modulation of epithelial responses rather than epithelial loss leading to decreased expression.

There is growing evidence that VEGF plays a supportive role in disease processes and that certain levels are necessary for cellular proliferation and differentiation. *In vitro* studies indicate that VEGF has a positive effect on epithelial cellular proliferation and can increase cell survival via reduction of caspase-3 in apoptosis [42, 43]. At day 4 post-
infection there was enhanced cellular proliferation in RSV infected lambs pretreated with rhVEGF. Interestingly, by day 6 proliferation markedly decreased in this group. We proposed that an increase in cellular proliferation in RSV infected lambs pretreated with rhVEGF would lead to enhanced lesion resolution. We initially saw an increase in proliferation at day 4 but not at day 6. However, lambs pretreated with VEGF had a marked decrease in pulmonary pathology at day 6 indicating that repair may have occurred at an earlier time point as may be suggested by the higher proliferation at day 4. At day 6, RSV infected lambs pretreated with PBS had high cellular proliferation, likely due to the severity of RSV infection and the response to repair the injury.

Intrapulmonary treatment with rhVEGF at the concentration used in this study resulted in minimal pulmonary pathology. Administration of rhVEGF has been reported to cause mild increase in monocyte recruitment early after administration [44]. In a preterm mouse model of lung prematurity, intratracheal administration of rhVEGF resulted in increased surfactant protein production with no adverse effects on the lungs [29]. There is a report of pulmonary hemorrhage and hemosiderosis in a mouse model over-expressing VEGF; however VEGF protein was far higher than physiologic levels [45]. The lack of major adverse side effects with intrapulmonary administration makes VEGF a possible therapeutic candidate.

The effect of rhVEGF pretreatment on viral infection is likely multi-factorial with effects on cellular proliferation and alteration of epithelial gene expression as described for SP-A and SBD-1. In addition, VEGF is a monocyte/macrophage chemoattractant [44, 46] via VEGF R-1. Macrophages not only internalize cellular debris but also modulate immune
responses in the lung by secretion of cytokines that are important for activation of CD8+ lymphocytes and antiviral responses [47]. Recruitment of macrophages may aid in the decreased lesions and virus that were observed in animals pretreated with rhVEGF. A recent study demonstrated that VEGF serves an immunostimulatory role by increasing the expression of IL-8, TNFα, and IFNγ within 24 hours of administration [48]. In our study, VEGF increased TNFα with no effect on IL-6 and IL-8.

This study shows that pretreatment with rhVEGF prior to RSV infection decreased viral mRNA and decreased pulmonary lesion severity at peak infection (day 6) as compared to RSV animals that did not receive rhVEGF. In addition, rhVEGF increased SP-A and SBD-1 expression exhibiting regulation of key epithelial innate immune genes. Pretreatment with rhVEGF altered cellular proliferation in RSV infected lambs at 4 and 6 days post-infection. There are likely other pulmonary effects induced by administration of rhVEGF in addition to those investigated here, that may provide interesting insight into disease pathogenesis and further the development of novel therapeutics.

Acknowledgements

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
References


Tables:

**Table 1:** Treatment groups for assessing VEGF pretreatment in RSV disease

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=4 (days 2, 4) n=6 (day 6)</td>
<td>PBS</td>
</tr>
<tr>
<td>n=4 (days 2, 4) n=6 (day 6)</td>
<td>PBS</td>
</tr>
<tr>
<td>n=4 (days 2, 4) n=6 (day 6)</td>
<td>VEGF</td>
</tr>
<tr>
<td>n=4 (days 2, 4) n=6 (day 6)</td>
<td>VEGF</td>
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Table 2. Primers and probe sequences for ovine gene expression assessed by real-time qPCR

<table>
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<th>Gene</th>
<th>Fwd:</th>
<th>Rev:</th>
<th>Probe:</th>
</tr>
</thead>
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<td>RSV</td>
<td>5'GCTCTTAGCAAGTGCAAGTTGA</td>
<td>5'TGCTCCGTTGGATGTTATT</td>
<td>5'-6FAM-ACACTCAACAAAGATCAAATCTGTCATCCACGCT-TAMRA</td>
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<tr>
<td>SP-A</td>
<td>5'TGACCCCTATGGCTCCTCTGGAT</td>
<td>5'GGGCTTCCAAGACAAACTTCCT</td>
<td>5'-6FAM-ACGTTCAGCTGAGAAT</td>
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<tr>
<td>SP-D</td>
<td>5'ACGTTCAGCTGAGAAT</td>
<td>5'GGTGTTGACCATTTTCTAGGAGCTC-TAMRA</td>
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</tr>
<tr>
<td>SBD1</td>
<td>5'GCTGTCTCTGGTGAACCTTC</td>
<td>5'GGTGTTGACCATTTTCTAGGAGCTC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>Rev: 5'TTCCAAGCTGCTGTGCT</td>
<td>5'TTGACAGAAACCTGACCTCCACA</td>
<td>5'-6FAM-CCGAGCAGGTCGCTAGACATGA-TAMRA</td>
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<tr>
<td>IL-6</td>
<td>5'GCTGTCTCTGGTGATGACTTC</td>
<td>Rev: 5'TTGACAGAAACCTGACCTCCACA</td>
<td>5'-6FAM-CCGAGCAGGTCGCTAGACATGA-TAMRA</td>
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<td>Tumor necrosis factor α</td>
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<td>Rev: 5'CCTACAAGCTGGCTGGAGTCGAG</td>
<td>5'-6FAM-CAAGGGCACAGTGCTACCAACGAA-TAMRA</td>
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<tr>
<td>Interferon β</td>
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<td>5'CTCTCAGAGGAGCCTACCAACGAA-TAMRA</td>
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<td>VEGF</td>
<td>Fwd: 5'GCCCACTGAGGAGCTTCAACATC</td>
<td>Rev: 5'GGGCTTCTCCTGTGGTTCTTTC</td>
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<td>VEGF Receptor-1</td>
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<td>5'-6FAM-CTGCGGAGGAGGAGAGAGAGAGAGAGAGAGAGAG -MGBNFQ</td>
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</table>
| VEGF Receptor-2 | Fwd: 5'CCAGTGGGCTTCAGAAGCAGAGAAATAATCAG | Rev: 5'GCAACAAAACGCTTTCATGT   | 5'-6FAM-CTGCGGAGGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
| Ubiquitin     | Fwd: 5'GTTGGCTGTATTACATTCTTCAAGAGTAC       | Rev: 5'AAATGGCTAGAGTGCAGAACGAT | 5'-6FAM-TTCTAATGCTGAGTGGATG-A-MGBNFQ |
| OvRPS15       | Fwd: 5'CGAGATGTTGCGGCAGACAT | Rev: 5'GCTTGATTTCCACCTGGTTGA | 5'-VIC-CCGGCGCTCTACAACGGCAGGACC-TAMRA |

6FAM or VIC, 5’ Fluorescent reporter dye; TAMRA, Fluorescent quencher dye; MGBNFQ, Minor groove binding non-fluorescent quencher
Figure 1: Photomicrograph of lung from an RSV infected lamb pretreated with PBS (A) and RSV infected lamb pretreated with VEGF (C). RSV infected lambs pretreated with PBS had suppurative bronchiolitis with locally extensive alveolar consolidation and infiltration of lymphocytes and plasma cells. RSV infected lambs pretreated with VEGF had decreased alveolar consolidation, bronchiolitis and inflammatory cell infiltration. RSV antigen staining by immunohistochemistry demonstrated multifocal immunoreactive cells within the
bronchiolar epithelium, airway lumen and interstitium (B). Lambs pretreated with VEGF had decreased antigen at day 6 post-infection (D). Bar =100µm

Figure 2: Pretreatment with exogenous rhVEGF decreased histologic consolidation score indicating a decrease in alveolar consolidation and inflammatory cell infiltration (*p<0.05).
Figure 3: At day 2, lambs pretreated with rhVEGF had significantly higher RSV mRNA levels (A). At day 4, RSV mRNA levels in the rhVEGF pretreated lambs were decreasing (B) with RSV mRNA expression levels significantly lower than PBS pretreated lambs at 6 days post-infection (C) (*p<0.05, **p<0.01).
Figure 4: Pretreatment with exogenous rhVEGF and inoculation with RSV altered expression of surfactant protein A. At day 4, administration of rhVEGF increased SP-A expression in non-RSV infected lambs, as compared to PBS pretreatment. Inoculation with RSV increased SP-A in both control and rhVEGF pretreated groups (B). At day 6, SP-A expression in rhVEGF pretreated non-RSV infected lambs remained increased. SP-A expression was decreased in RSV infected lambs that were pretreated with rhVEGF compared to those that were pretreated with PBS (C) (*p<0.05, **p<0.01, ***p<0.001).
Figure 5: Pretreatment with exogenous rhVEGF and inoculation with RSV altered expression levels of sheep beta-defensin-1 (SBD-1). At day 2, inoculation with RSV increased SBD-1 levels in both pretreatment groups however SBD-1 expression was higher in animals pretreated with rhVEGF (A). At day 4, administration of rhVEGF increased SBD-1 expression in non-RSV infected lambs, as compared to PBS control. SBD-1 expression in RSV infected animals remained increased (B). At day 6, SBD-1 expression in RSV infected lambs pretreated with rhVEGF was significantly decreased compared to RSV infected lambs pretreated with PBS. SBD-1 expression remained high in non-RSV infected lambs pretreated with rhVEGF (C) (*p<0.05, **p<0.01, ***p<0.001).
Figure 6: Pretreatment with exogenous rhVEGF and inoculation with RSV altered expression levels of TNFα. At day 2, TNFα expression was significantly increased in RSV infected lambs in both pretreatment groups (A). At day 4, TNFα expression increased in non-RSV lambs pretreated with rhVEGF. Expression decreased from day 2 levels in RSV infected lambs with higher expression in lambs pretreated with rhVEGF (B) (′p<0.05, **p<0.01, ***p<0.001).
IL-8

**Figure 7:** RSV infection increased expression of IL-8 in both pretreatment groups (*p*<0.05).
Figure 8: At day 4, VEGF expression levels are increased in RSV infected lambs in both pretreatment groups. At day 6, VEGF expression levels are decreased in RSV infected animals in both pretreatment groups ($^*p<0.05$, $^{***}p<0.001$).
**Figure 9:** Photomicrograph of immunohistochemical staining for Ki-67 antigen staining to measure cellular proliferation. Immunoreactive cells are present multifocally within the bronchiolar epithelium and within the interstitium (A,B). Bar =100µm
Figure 10: Quantification of Ki-67 immunoreactive cells within lung tissue from lambs in all groups. At day 4, RSV infected lambs pretreated with VEGF had increased numbers of Ki-67 immunoreactive cells compared to VEGF control. At day 6, RSV lambs pretreated with PBS had increased number of KI-67 immunoreactive cells compared to PBS control. RSV infected lambs pretreated with rhVEGF had no significant increase in Ki-67 immunoreactivity (*p<0.05, ***p<0.001).
CHAPTER 4: DIFFERENTIAL EXPRESSION OF VEGF ISOFORMS AND VEGF RECEPTORS DURING ONTOGENY OF OVINE LUNG

A paper submitted for publication in American Journal of Physiology – Lung Cellular and Molecular Physiology

Alicia K. Olivier, Jack M. Gallup, Albert van Geelen, Mark R. Ackermann

Abstract

Vascular endothelial growth factor (VEGF) is a mediator of angiogenesis with multiple functions in lung development and homeostasis. The purpose of this study was to define developmental expression of VEGF-A and its receptors in ovine lung. VEGF mRNA and protein expression was measured in lung tissue from prenatal (115 and 130 days gestation), term, postnatal (15 days post-birth) and adult sheep. Isoform-specific expression (VEGF\(_{120}\), VEGF\(_{164}\), VEGF\(_{188}\) and VEGF\(_{120b}\)) was measured by reverse transcriptase PCR to determine if developmental patterns were present. VEGF mRNA was most abundant at 115 and 130 days gestation with low expression at term and in adult lung. VEGF mRNA expression at 15 days post-birth was similar to prenatal expression levels. During prenatal development, angiogenic isoforms were highly expressed (VEGF\(_{120}\), VEGF\(_{164}\), and VEGF\(_{188}\)) and significantly decreased at term. VEGF receptor-1 was highly expressed early in development with decreased expression at term and 15 days post-birth. VEGF receptor-2 was highly expressed at 15 days post-birth. VEGF protein levels were low in prenatal lung but high in adult lung, which did not correlate to mRNA expression. VEGF\(_{120b}\) was the only anti-angiogenic isoform detected by western blot indicating a possible role in development.
These results show that VEGF and VEGF receptors are differentially expressed during ovine pulmonary development and that mRNA levels do not correlate with protein expression.

**Introduction**

Vascular endothelial growth factor (VEGF) is a well-known endothelial cell mitogen that regulates endothelial cell differentiation in physiologic and pathologic angiogenesis. VEGF is also a potent mediator of permeability important in inflammatory processes (13). VEGF has additional non-endothelial functions, such as epithelial cell proliferation and macrophage activation. In adults, VEGF is expressed in almost every tissue which underscores its vital nature (23).

Expression of VEGF and VEGF receptors is present in bronchiolar epithelial cells, type II epithelial cells and alveolar macrophages (4, 6, 16, 24). Studies demonstrate that pulmonary VEGF is important for the survival and growth of both endothelial and epithelial cells (37). Decreased VEGF or VEGF receptor signaling in the lung leads to caspase-3 dependent apoptosis of septal endothelial and epithelial cells resulting in emphysematous change (21, 34). VEGF also functions in surfactant homeostasis and can upregulate surfactant protein production (11).

The VEGF family consists of five members, VEGF-A, -B, -C, -D and placental growth factor. VEGF-A is the most widely studied. The VEGF-A (VEGF) gene is organized into eight exons that are alternatively spliced to form different polypeptide isoforms (16). There are two families of VEGF isoforms, the pro-angiogenic isoforms (VEGF<sub>xxx</sub>) formed by proximal splice site selection in the terminal exon (exon 8) and the
anti-angiogenic isoforms (VEGF_{xxb}) generated by an alternate distal splice site, which results in a six amino acid difference at the carboxyl terminus (28). For every angiogenic isoform there appears to be an inhibitory isoform that functions in modulating angiogenic functions. VEGF_{121, 165} and 189 are the three main pro-angiogenic isoforms in humans. Less frequently reported isoforms include VEGF_{145} (30), VEGF_{162} (22), and VEGF_{183} (20). Mouse VEGF-A isoforms have one less amino acid than the human isoforms (VEGF_{120, 164, 188}) (33) with sheep isoforms similar to those (VEGF_{120, 164, 187}) (A. van Geelen, unpublished observation). VEGF isoforms have different receptor binding properties and location within the extracellular matrix (ECM). VEGF_{189} binds heparin with high affinity and is therefore sequestered within the ECM. VEGF_{121} does not bind heparin and is a freely diffusible protein. VEGF_{165} has intermediate binding properties and is both diffusible and bound to an extent to the cell surface and ECM (14). The functions of the anti-angiogenic isoforms (VEGF_{xxb}) have not been completely characterized. High expression of anti-angiogenic isoforms have been found in non-angiogenic tissues such as the colon (36) and vitreous humor of the eye (29) with decreased expression in angiogenic conditions (28). A recent study examined expression of VEGF_{xxb} in normal human adult lung and lung from patients with acute respiratory distress syndrome (ARDS) (35). This study found significant VEGF_{xxb} protein expression in normal lung, primarily in alveolar epithelial cells and macrophages, with minimal expression in ARDS lung. This study indicates that VEGF_{xxb} isoforms are significantly expressed in normal lung and likely have important homeostatic functions.
Expression of VEGF and VEGF receptors are critical to embryonic development. Deletion of a single VEGF allele (8, 15) or either of the VEGF receptors -1 (Flt-1) or -2 (Flk-1) (16), results in embryonic lethality. Mice expressing only VEGF$_{120}$ survive to term but die soon after birth with vascular defects and altered endothelial cell distribution (9, 32). Embryonic over-expression of VEGF is also embryonic lethal, resulting in severe cardiac vascular abnormalities (25).

In this study we measured the expression of VEGF mRNA and protein during lung development and in adult lung. Expression was measured in prenatal (115 and 130 days gestation), term, postnatal (15 days post-birth) and adult lung. Isoform-specific expression (VEGF$_{120}$, VEGF$_{164}$, VEGF$_{188}$ and VEGF$_{120b}$) was also measured to determine if isoform expression patterns were present during various stages of pulmonary development.

**Materials and Methods**

**Tissue Collection:**

Tissues used in this study were stored from previous studies. Lung tissue was collected from four fetuses at 115 days gestation (145 days is term), four fetuses at 130 days gestation, four term neonates (6 hours following natural birth), four lambs at 15 days post-birth and four adults (>3 years of age). Samples were collected and placed in cassettes then in 10% neutral-buffered formalin for immunohistochemical analysis. Additional lung samples were snap-frozen on dry ice for PCR assays. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at Iowa State University.

**VEGF Immunohistochemistry:**
Immunohistochemical staining was performed for VEGF antigen (clone C-1, 200 µg/ml used at 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA). Paraffin-embedded lung sections were cut at 5 µm thickness onto positively charged slides. Slides were incubated at 58°C for 25 minutes, deparaffinized in xylene, rehydrated in graded ethanol washes and washed in phosphate-buffered saline (PBS). Antigen retrieval was performed in TRIS/EDTA (pH 9.0) for 15 minutes in a steamer, then cooled for 20 minutes to room temperature. Slides were then rinsed with phosphate-buffered saline (PBS). Nonspecific binding was blocked by incubation in 10% normal goat serum in TRIS/PBS with 3% bovine serum albumin (BSA). Following overnight incubation with the primary antibody (in TRIS/PBS/BSA) the slides were rinsed then incubated with a biotinylated anti-mouse antibody (KPL, Gaithersburg, MD) at a dilution of 1:200. To control for nonspecific staining, a control slide was processed with omission of the primary antibody. Following the secondary antibody, the slides were incubated for 20 minutes in a 3% peroxidase block then incubated with a peroxidase-conjugated streptavidin (1:200) for 45 minutes (Invitrogen, Carlsbad, CA). After three PBS washes, the color was developed with Nova Red (Vector). The slides were then counterstained with Harris’ hematoxylin, dehydrated and cover-slipped.

Immunohistochemistry scores were based on percent epithelial cell staining within bronchioles and staining intensity. The percent of epithelial cells staining in bronchi was graded as follows: 1, 0-25%; 2, 25%-50%; 3, 50%-75%; and 4, 75%-100% from 10, 20X fields. Tissues were originally screened to identify staining intensity patterns. Staining intensity was graded as follows: 1, weak (faint brown); 2, moderate (brown); 3, intense (dark
brown/black). The values for percent epithelial staining and staining intensity were combined to give an immunohistochemical score for each animal.

**RNA Analysis**

Total RNA was isolated from whole lung tissue via Trizol according to manufacturer’s guidelines (Invitrogen, Carlsbad, CA). Following RNA isolation, the pellets were resuspended in nuclease free water and heated at 65°C for 5 min.

**One-Step Real-Time qPCR:** RNA samples were assessed for quantity and purity by spectrophotometry at 260nm and 280nm and were DNase treated (TURBO DNase, Ambion) and diluted 1:10 with nuclease-free water for one-step real-time qPCR. Reactions were carried out using SuperScript™ III Platinum® One-Step quantitative RT-PCR System with ROX kit (Invitrogen, Carlsbad, CA). A test plate was run using a mixture of all RNA samples serially diluted, to determine the optimal dilution range for each target standard curve and individual sample dilutions. Following determination of the optimal dilution range for each target, samples and standards were run in duplicate (18). Ovine primers and probe were used to detect VEGF, VEGF R-1 and VEGF R-2. The primers and probe for VEGF detected VEGF isoforms₁₂₀,₁₆₄, and₁₈₇ (Table 1). Each target gene amplification was converted to a relative quantity using a target specific standard curve and normalized to two endogenous reference genes, ovine ubiquitin and ovine ribosomal S15 (17).

**Gel Electrophoresis:** Using the 260nm absorbance readings the samples were diluted to the same RNA concentration (111 ng/μl). Total RNA was reverse transcribed using SuperScript™ III (Invitrogen, Carlsbad, CA) and random pentadecamers (Integrated DNA
Technologies, Coralville, IA). The following primer set was used to amplify the cDNA for ovine VEGF products: VEGF forward: 5'-GCCAGCACATAGGAGAGATGAG, VEGF reverse: 5'-TGTTCTGTCGATGGTGGTGGTG. These primers were predicted to produce PCR products of 166bp, 278bp, 370bp, 436bp for sheep VEGF isoforms 120b, 120, 164, and 187 respectively. PCR amplification of cDNA was performed in an Eppendorf Mastercycler using TaKara Ex Taq (Takara, Madison, WI). PCR products were separated in a 2% agarose/TAE with GelRed stain (Biotium, Hayward, CA). Imaging and densitometry was performed using an Alpha Innotech Imager with AlphaEaseFC software.

Vascular endothelial growth factor (VEGF) protein analysis by ELISA

Whole lung was homogenized on ice in T-PER (Tissue Protein Extraction Reagent, Pierce, Rockford, IL) along with a Complete TM Protease Inhibitor Cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). The TPER directions were followed using 0.5 grams of lung tissue to 5 ml of TPER reagent. Supernatants from the homogenization were assayed using a VEGF-A enzyme-linked immunosorbent assay, which recognizes VEGF$_{120}$ and VEGF$_{165}$ (Invitrogen, Carlsbad, CA). Samples were run in duplicate and expressed as picograms VEGF per gram of lung tissue.

Western blotting

Whole lung from each animal was homogenized on ice in TPER along with a cocktail of protease inhibitors. Protein concentration was determined using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Samples were resuspended in sodium dodecyl sulfate (SDS) sample buffer and heated to 100ºC for 5 minutes, and resolved by 8-
16% polyacrylamide gels (Pierce, Rockford, IL). After electrophoresis, proteins were transferred to a polyvinylidene fluoride Immobilon-P membrane (Millipore, Bedford, MA) and blocked with 3% bovine serum albumin in PBS 0.05% Tween 20 (PBST) for one hour at room temperature. After blocking, membranes were incubated overnight at 4°C with the specified primary antibody: mouse monoclonal anti-VEGF (clone VG-1, 1:2000) (Millipore, Bedford, MA), mouse monoclonal anti-VEGFxxxb (MAB3045 at 1:2000) (R&D systems, Minneapolis, MN), or anti-β-actin (1:10,0000) (Sigma, St. Louis, MO). After 3 washes in PBST, membranes were incubated with an Alexa Fluor 488 conjugated goat anti-mouse secondary antibody (1:2000) (Invitrogen, Carlsbad, CA). After 3 washes in PBST, fluorescent signal was detected by a Typhoon 8600 imaging system (GE Healthcare, Quebec, Canada). When necessary, membranes were stripped using a stripping buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 50°C for 30 min.

**Sequencing:**

PCR products were cloned into pCR4TOPO sequencing vector (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Colonies resulting from the TOPO cloning reactions were screened for VEGF inserts by performing colony PCR using the primers for the splice isoform screening. Positive clones were cultured overnight and plasmid DNA was isolated the next day and submitted for sequencing to the Iowa State University DNA sequencing facility.

**Statistical Analysis**
Statistical analyses were performed with commercially available statistics software (GraphPad Prism, GraphPad Software, San Diego, CA). Data was analyzed for normal distribution and log transformed when necessary. Comparisons were done using one-way ANOVA followed by Tukey’s post-test analysis. Immunohistochemical data was analyzed using a Kruskal-Wallis test and a Dunn multiple-comparison posttest. Data are represented as means (±SEM). A $P$ value <0.05 was considered significant.

Results

**VEGF and VEGF receptor mRNA expression**

VEGF mRNA expression was highest at 115 days gestation with a decrease in expression at 130 days gestation and term (Figure 1). Expression increased at 15 days post-birth but was low in adult lung. VEGF R-1 expression in the lung was high at 115 days gestation but decreased at term and was lowest at 15 days post-birth (Figure 2). Expression of VEGF R-1 in adult lung was similar to expression during prenatal development. VEGF R-2 expression was low at 115 days gestation with increased expression at 15 days post-birth (Figure 3). Expression of VEGF R-2 in adult lung was similar to expression levels at 130 days gestation and term.

**VEGF isoform expression**

Expression of VEGF isoforms; VEGF$_{120}$, VEGF$_{164}$, VEGF$_{187}$ and VEGF$_{120b}$ were measured in lung at 115 days gestation, 130 days gestation, term and adult via PCR amplification (Figure 4 A). VEGF$_{120}$ was highly expressed at 115 days and 130 days gestation compared to term and adult (Figure 4 B). VEGF$_{164}$ was highly expressed at 115
and 130 days gestation with low expression at all other time-points (Figure 4 C). VEGF\textsubscript{187} was highly expressed at 130 days gestation with a decrease at term and in adult lung (Figure 4 D). VEGF\textsubscript{120b} did not change significantly (Figure 4E). VEGF\textsubscript{187} was the predominant isoform at all developmental time points but not in the adult lung (Figure 5). VEGF\textsubscript{120b} was the predominant isoform in adult lung.

\textbf{VEGF immunolocalization and protein concentration}

VEGF antigen was localized by immunohistochemistry to the cytoplasm of the bronchiolar epithelial cells lining the small and medium but not the large bronchioles in all age groups. In adult lung, multifocal alveolar macrophages were immunoreactive for VEGF antigen. Lung at 115 and 130 days gestation had multifocal immunoreactive bronchiolar epithelial cells with weak epithelial staining intensity (Figure 6 A, B). At term and 15 days post-birth there were increased number of immunoreactive cells with increased staining intensity (Figure 6 C, D). In the adult lung, VEGF antigen was present within the majority of bronchiolar epithelial cells with intense staining (Figure 6 E). An immunohistochemical score was given to each section of lung based on the percentage of immunoreactive epithelial cells and staining intensity. Adult lung had the highest immunohistochemical score and the lowest score was in lung from 115 and 130 days gestation (Figure 6 G).

VEGF\textsubscript{187} and VEGF\textsubscript{164} were detected consistently in lung tissue by western blot, indicating that they are the predominant isoforms in the developing ovine lung (Figure 7 A). VEGF protein concentration quantified by ELISA demonstrated high VEGF concentrations in adult lung (Figure 7 B). VEGF protein was also higher at 15 days post-birth compared to 115 days gestation. VEGF\textsubscript{120b} was detected by western blot and demonstrates that this anti-
angiogenic isoform is significantly expressed throughout pulmonary development (Figure 7 C).

**Discussion**

VEGF expression in the lung is essential for regulating vasculogenesis during development. VEGF also functions in pulmonary homeostasis following birth and has been shown to regulate surfactant protein production (10, 11), reduce apoptosis of epithelial cells (31) and may also serve to promote reparative processes following injury (7, 12, 26). During ovine lung development, the angiogenic VEGF isoforms (VEGF\textsubscript{120}, VEGF\textsubscript{164}, and VEGF\textsubscript{187}) followed the same pattern – high mRNA levels during prenatal development with a significant reduction in expression at term. Differential pulmonary VEGF isoform expression during development has been documented in the mouse, rat and rabbit. In the rat and mouse there is an increase in VEGF\textsubscript{188} mRNA prior to birth with a moderate decrease following birth, similar to the lamb (19, 27). In the mouse, VEGF\textsubscript{120} and VEGF\textsubscript{164} decrease prior to birth and remain low 30 days following birth, differing slightly from ovine lung in which VEGF\textsubscript{120} and VEGF\textsubscript{164} mRNA decreased at term. In the rabbit lung, VEGF\textsubscript{165} was highly expressed in late fetal development but decreased significantly by 10 days after birth, similar to the lamb (39). VEGF\textsubscript{189} expression in the rabbit was high in late fetal lung development and remained high following birth, while in the lamb there is marked decrease of VEGF\textsubscript{187} following birth. In ovine lung VEGF\textsubscript{120b} expression remained consistent throughout development. To our knowledge VEGF\textsubscript{120b} has not been characterized during pulmonary development in any other species and because of its inhibitory activity may have a key role in VEGF regulation. The similarities of VEGF isoform expression in rodent,
rabbits and in lambs in this study, suggests that developmental regulation of VEGF isoform expression in the lung appears to be conserved.

There was some inter-animal variability in isoform expression with the term, 15 days post-birth and adult groups. The reason for the variability is unknown, however there may be several factors involved. At the time of birth animals must expand their lungs and begin breathing on their own. Stress or dystocia at parturition could affect pulmonary inflation and gas exchange, which may then alter isoform expression. Also at the time of birth, animals are exposed to new environmental airborne substances including particulate material, vapors, aerosols, alterations in humidity, microbial products, fumes and other factors that may change normal lung homeostasis and alter isoform expression. Lastly, lambs used in the study are out-bred, therefore genetic factors such as single nucleotide polymorphisms in the genes themselves or regulatory elements could also play a role in expression levels following birth.

Although VEGF mRNA expression was high during prenatal lung development, with decreasing levels after birth, VEGF protein levels were low prenatally and increased towards adulthood. Protein levels were highest in adult sheep, in which there was low VEGF mRNA expression. It is well-known that VEGF transcriptional and translational regulation is complex and controlled at many levels. Hypoxia is the most studied inducer of VEGF transcription and mRNA stablility (38) however other factors such as growth factors, hormones, and oncogenes activate VEGF transcription (14, 16). Transcriptional regulation occurs at the two promoter regions; promoter one (P1) is located at the 5’-untranslated region (5’UTR) and a second internal promoter is located 633 nucleotides downstream from P1 (2).
Translation is controlled by the classical AUG initiation codon and alternatively by three CUG start codons located within the 5′UTR. Translation can also be regulated by two internal ribosome entry sites (IRES A and IRES B) located upstream of the AUG start codon. Regulation of VEGF specific isoform expression is largely unknown. One study demonstrated that the RNA structure of alternatively spliced mRNA controls selection of the translation initiation codon (5). Another study demonstrated an upstream ORF within the IRES A that acts as a regulatory element to negatively control isoform (VEGF121) expression (3). A limitation of this study was the inability to quantify isoform specific protein expression, due to the lack of isoform-specific antibodies.

The anti-angiogenic isoform, VEGF120b, was detected via western blot; however the functional role of this and other anti-angiogenic isoforms in pulmonary development is unknown. Interestingly, VEGF120b was the only anti-angiogenic isoform detected in ovine lung tissue even though the blot was probed with an antibody that should recognize the carboxyl terminus of all anti-angiogenic isoforms. It is possible that VEGF164b is not highly expressed and below the limit of detection, indicating that VEGF120b may play a more significant role in pulmonary development. A recent study examining the protein expression of VEGFxxxb in normal adult lung and adult lung from ARDS patients demonstrated expression of both VEGF121b and VEGF165b (35). However, the signal from VEGF121b was much more intense, indicating that VEGF121b appears to be more prevalent in lung than VEGF165b. Further studies are necessary to examine the role of anti-angiogenic isoforms in pulmonary development.
VEGF concentrations are critically important in lung development, and rodent models overexpressing VEGF in the lung demonstrate lung dysmorphogenesis (1). Therefore, the low VEGF protein concentration in the ovine lung during prenatal development is likely due to tight regulation at the translational level to maintain adequate concentrations necessary for development. Several hypotheses can be made regarding the low VEGF mRNA expression and high protein concentration in the adult lung. One hypothesis may be that adult animals have more efficient translation at the IRESes, leading to higher protein expression without an increase in mRNA expression. Another hypothesis is that binding of the larger isoforms via their heparin-binding sites to the ECM allows VEGF to be present when necessary, without increased mRNA expression.

Differential expression of the VEGF receptors was also present during development. Expression of VEGF R-1 decreased following birth but increased in adult lung. The effects of VEGFR-1 are varied depending on developmental stage and cell type (16). VEGF R-2 is the major mediator of the mitogenic and permeability effects of VEGF. Expression of VEGF R-2 was low during prenatal development but increased at 15 days post-birth. Expression was also low in adult lung. Receptor expression during development is extremely important for proper vascular development, however the reason for high VEGF R-2 expression at 15 days post-birth is not known. It could be due to the fact that at this stage of life, the lung has a converging set of demands that include: growth and further development, function (airflow and gaseous exchange), and adaptation to newly encountered environmental substances within the air.
This study demonstrates that expression of the major VEGF isoforms are differentially regulated during ovine pulmonary development and in the adult lung. Importantly, the expression of mRNA diverged remarkably from that of the protein profile. Identifying isoform specific protein levels of antigogenic (VEGF\textsubscript{xxx}) and anti-angiogenic (VEGF\textsubscript{xxxb}) isoforms will aid in the understanding of the role of specific isoforms during development. Differential isoform expression and the divergent mRNA and protein levels demonstrate the importance and complexity of VEGF regulation in proper pulmonary development and homeostasis.

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References


Table 1: Primers and probe sequences for ovine gene expression assessed by one-step real-time qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fwd</th>
<th>Rev</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>5'-GCCCACTGAGGAGTTCAACATC</td>
<td>5'-TGGCTTTTGGTGAGGGTTGATC</td>
<td>5'-6FAMCCATGCAGATTATG-MGBNFQ</td>
</tr>
<tr>
<td>VEGF Receptor-1</td>
<td>5'-TCAGGTGAGCTTTGGACAAAAATG</td>
<td>5'-CACCCTGCGAGACAGCTCTCA</td>
<td>5'-6FAM-CTGCCAAGAGGAGAG-MGBNFQ</td>
</tr>
<tr>
<td>VEGF Receptor-2</td>
<td>5'-CCAGTGGGGCTGATGACCAA</td>
<td>5'-GCAACAAAAACGGCTTTTCATGT</td>
<td>5'-6FAM-AAGAACAGCAGCGAGTTCCGCGG-TAMRA</td>
</tr>
<tr>
<td>OvRPS15</td>
<td>5'-CGAGATGGTGCCAACGACAT</td>
<td>5'-GCTTGATTTCCACCTGGTTGA</td>
<td>5'-VIC-CGGCGTCTACAACGGCAAGACC-TAMRA</td>
</tr>
<tr>
<td>Ubiquitin (ovine)</td>
<td>5'-GGTGGCTGTAAAATCTTCAG</td>
<td>5'-AAATGGCTAGAGTGCAAGACGAT</td>
<td>5'-6FAM-TTCATAATGCTCAGTGATCG-MGBNFQ</td>
</tr>
</tbody>
</table>

6FAM or VIC, 5’ Fluorescent reporter dye; TAMRA, Fluorescent quencher dye; MGBNFQ, Minor groove binding non-fluorescent quencher
Figure 1: VEGF mRNA expression was highest in prenatal lamb lung at 115 and 130 days gestation and at 15 days post-birth. The lowest expression of VEGF mRNA was in term lamb lung and adult lung. (DG= days gestation; PB = post-birth) *p<0.05, ***p<0.001.
**Figure 2:** VEGF R-1 mRNA expression was highest at 115 days gestation and decreased at term. The lowest expression was at 15 days post-birth. Expression in adult lung was similar to levels present during prenatal development. (DG = days gestation; PB = post-birth) (*$p<0.05$, ***$p<0.001$).
Figure 3: VEGF R-2 mRNA expression in the lung was low at 115 days and 130 days gestation and at term. Expression was highest in the lung at 15 days post-birth with expression in the adult similar to expression levels throughout prenatal development. (DG= days gestation; PB = post-birth) *p<0.05, ***p<0.001.
Figure 4: VEGF isoform expression in the lung during prenatal, postnatal, and adult lung.

Relative intensity of VEGF₁₂₀ (B), VEGF₁₆₄ (C), VEGF₁₈₇ (D), and VEGF₁₂₀b (E) derived from densitometry analysis of isoform expression as measured by PCR (A). There was high VEGF₁₂₀ expression at 115 days and 130 days gestation compared to term and adult. VEGF₁₆₄ was highly expressed at 115 and 130 days gestation with low expression in all other groups. VEGF₁₈₇ was highly expressed at 130 days gestation compared to term and adult. VEGF₁₂₀b did not change significantly through development and in adult lung. (DG= days gestation; PB = post-birth) *p<0.05, **p<0.01, ***p<0.001.
Figure 5: Percent relative expression of VEGF$_{120}$, VEGF$_{164}$, VEGF$_{187}$ and VEGF$_{120b}$ at 115 days gestation (DG), 130 days gestation, term, 15 days post-birth (PB) and adult lung. VEGF$_{187}$ was the predominant isoform expressed at all developmental time points but in the adult lung. VEGF$_{120b}$ was the predominant isoform expressed in adult lung.
Figure 6: Immunohistochemistry for VEGF antigen in the ovine lung at 115 days gestation (A), 130 days gestation (B), term (C), 15 days post-birth (D) and adult (E). Isotype control (F). VEGF immunoreactivity was present in the cytoplasm of bronchiolar epithelial cells lining the medium and small bronchioles. Multifocal alveolar macrophages were immunoreactive for VEGF antigen. The lungs were scored based on number of epithelial cells positive for the antigen and staining and staining intensity (G). Antigen staining at 115 and 130 days gestation had fewer immunoreactive epithelial cells and weaker staining intensity compared to term, 15 days post-birth and adult lung. (DG= days gestation; PB = post-birth)
A.

![Western Blot Image](image)

- **25 KDa**
- **VEGF<sub>187</sub>**
- **VEGF<sub>164</sub>**

- **β-actin**

- **115 DG**
- **130 DG**
- **Term**
- **15 PB**
- **Adult**

B.

**VEGF ELISA**

- **pg VEGF/1 gram lung tissue**

- **115 DG**
- **130 DG**
- **Term**
- **15 PB**
- **Adult**

- *******
- ****
- *****

C.

![Western Blot Image](image)

- **15 KDa**
- **VEGF<sub>120b</sub>**

- **β-actin**

- **115 DG**
- **130 DG**
- **Term**
- **15 PB**
- **Adult**
Figure 7: Western blot on lung tissue using an antibody against VEGF-A demonstrating expression of VEGF$_{164}$ and $187$ in developing lung (A). B. ELISA performed on ovine lung homogenates demonstrates VEGF protein expression was highest in adult lung with low expression at 115 days gestation, 130 days gestation and term. C. Western blot using an antibody against VEGF$_{xxxb}$ antibody demonstrates VEGF$_{120b}$ is expressed in developing and adult lung. (DG= days gestation; PB = post-birth) $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. 
CHAPTER 5: GENERAL CONCLUSIONS

RSV is the leading cause of bronchiolitis in infants and infects almost 100% of children by age 2. The only licensed therapy is a prophylactic monoclonal antibody used to treat infants at high risk but there is currently no licensed vaccine and no fully effective antiviral drug [1]. High risk infants include those born prematurely, infants with cardiopulmonary disease and infants with an immune deficiency [2, 3]. The innate immune response is very important in infants as their adaptive immune responses are immature and not fully effective [4]. The work in this dissertation: 1) characterizes a lamb model of RSV disease using the A2 strain of human RSV to develop an ovine model of RSV disease and to measure the epithelial innate immune response to RSV infection, 2) examines the effect of VEGF pretreatment on RSV disease severity and enhancement of the pulmonary innate immune response, and 3) defines developmental VEGF isoform and VEGF receptor expression in the ovine lung.

RSV Lamb Model

Although there are several animal models used to study RSV disease, there is no one model that fully replicates human pulmonary pathology and clinical signs. Mice are the most widely used animal model but are not very permissive to RSV infection. Pulmonary lesions in mice do not adequately represent human infection as pulmonary inflammation is centered on vessels, and not on airways as it is in humans [5]. Also, lung alveolar development, airway branching patterns, and airway cell content in mice differ from human and lambs [6]. The first study in this dissertation demonstrated that lambs were susceptible to a human RSV strain (strain A2) and exhibited clinical signs and pulmonary pathology similar to human
RSV disease. Pulmonary lesions included suppurative bronchiolitis with locally extensive alveolar consolidation and infiltration of lymphocytes and plasma cells with syncytial cell formation. Peak lesions were present at 6 days post-infection with lesion resolution at day 14. RSV antigen was present in the cytoplasm of bronchiolar epithelial cells, type II pneumocytes, and alveolar macrophages. RSV induced the expression of important epithelial innate immune genes, SP-A and SBD-1, demonstrating modulation of epithelial responses by the virus. In conclusion, infection with a human strain of RSV in the neonatal lamb reproduces the pulmonary pathology found in human infection and modulates epithelial innate immune responses. Replication and disease caused by this laboratory-adapted strain suggests that RSV strains with more virulence may induce enhanced disease. The lamb model will be beneficial for future investigations of pathogenesis, assessing viral virulence, and development of therapeutic compounds.

**Exogenous VEGF Administration Decreases RSV Disease Severity**

The second study examined the protective effect of VEGF pretreatment on RSV disease in the lamb model. VEGF is a well-known regulator of vasculogenesis that functions as an endothelial cell mitogen and vascular permeability factor in physiologic and pathologic angiogenesis [7]. In addition, VEGF is highly expressed in the lung and serves an essential role in pulmonary development, surfactant homeostasis and pulmonary repair processes [8, 9]. VEGF also increases surfactant protein production [10]. Surfactant protein is important in both lung maturation and innate immunity. Surfactant proteins-A and -D (SP-A and SP-D respectively) are collectins produced by epithelial cells and are found in the airway surface liquid [11]. Collectins modulate inflammatory responses within the lung to minimize
inflammatory-induced damage to maintain function. The collectins also aggregate and opsonize pathogens for enhanced clearance [12]. Mice deficient in SP-A and SP-D have decreased RSV clearance and increased pulmonary pathology as a result [13]. Human infants with polymorphisms in SP-A have increased RSV disease severity [14].

In this study, we hypothesized that VEGF would enhance the pulmonary innate immune response and thereby decrease RSV disease severity. Lambs were pretreated with human recombinant VEGF (rhVEGF) prior to RSV infection. Animals were sacrificed at 2, 4 and 6 days following infection to determine VEGF pretreatment effect on disease severity, alteration in important epithelial innate immune genes (SP-A, SP-D, sheep β-defensin-1), epithelial inflammatory products (TNFα, IFNβ, IL-6, IL-8) and endogenous VEGF and VEGF receptor expression. Pretreatment with rhVEGF decreased bronchiolitis, airway occlusion and alveolar consolidation at 6 days post-infection. Pretreatment with rhVEGF increased SP-A and SBD-1 expression exhibiting regulation of key epithelial innate immune genes by rhVEGF. RSV infected lambs pretreated with rhVEGF had increased cellular proliferation at 4 days post-infection. In conclusion, this study demonstrates that administration of rhVEGF prior to RSV infection significantly reduces RSV disease severity by decreasing pulmonary pathology, enhancing innate immune responses and increasing cellular proliferation.

**Differential Expression of VEGF and VEGF Receptors Throughout Development**

The third study measured the expression of VEGF and VEGF receptors during ovine pulmonary development and in the adult ovine lung in order to define differential developmental expression patterns. VEGF is necessary for proper embryologic development
and lung development. Mice lacking even one allele of VEGF die during embryonic
development with defects in vascular formation [15, 16]. When VEGF is inhibited during
lung formation, epithelial cell proliferation halts and cells undergo apoptosis [17]. VEGF
pre-mRNA is spliced into different mRNA molecules yielding various polypeptide isoforms
that are either angiogenic (VEGF_{xxx}) or anti-angiogenic (VEGF_{xxxb}) [18]. VEGF and VEGF
receptors are expressed by bronchiolar epithelial cells, type II epithelial cells and alveolar
macrophages [19, 20]. Pulmonary functions of VEGF include structural development,
surfactant homeostasis and pulmonary reparative process, as previously mentioned.

In this study, the mRNA for expression of the angiogenic VEGF isoforms; VEGF_{120},
VEGF_{164}, and VEGF_{187} and one anti-angiogenic isoform, VEGF_{120b}, were measured at 115
days gestation, 130 days gestation, term, 15 days post-birth and in adult lung. Overall, there
was high expression of the angiogenic isoforms prior to birth with a decrease at birth. The
anti-angiogenic isoform, VEGF_{120b}, did not change significantly throughout development.
Interestingly, VEGF_{187} was the predominant isoform present during all developmental
periods except the adult lung, in which VEGF_{120b} was the predominant isoform. VEGF
receptors-1 and -2 were also differentially expressed. Protein concentrations (VEGF_{164} and
VEGF_{187}) were high in adult lung but low at all other developmental periods, significantly
diverging from mRNA expression levels. In conclusion, this study demonstrates that
expression of the major angiogenic VEGF isoforms are differentially regulated during ovine
pulmonary development and in the adult lung. High VEGF mRNA expression did not
correlate to increased protein concentration, signifying the importance and complexity of
VEGF transcriptional and translational regulation in normal pulmonary development and homeostasis.

**Future Research Directions**

These studies demonstrate that pretreatment with rhVEGF decreased RSV pulmonary pathology and enhanced epithelial innate immune responses. Future studies should address rhVEGF administration after RSV infection or disease onset, to determine if rhVEGF could have therapeutic potential in patients already exhibiting clinical signs. These studies addressed VEGF’s effect on epithelial innate gene expression; however VEGF has other functions that should be investigated, such as macrophage chemoattraction that may serve to modulate the immune response to infection and its ability to increase vascular leakage of serum from vessels. The mechanistic basis by which rhVEGF reduces severity of RSV infection is likely multifactorial. *In vitro* studies could be used to address its effects on epithelial protection and viral replication.

We also demonstrated that expression of VEGF during pulmonary ontogeny is developmentally regulated. Future experiments should determine if there are changes in VEGF isoform expression during RSV infection that may alter pulmonary development and homeostatic functions. Alteration in expression should then be confirmed at the protein level, if and when antibodies are available for the specific isoforms. Identification of isoform-specific alteration during RSV infection may be beneficial for therapeutic design and will aid in furthering our understanding of the role of specific isoforms during infection. This information may be helpful not only in preventing or reducing severity of RSV infection; but perhaps also in enhancing lung maturity in premature infants.
References


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