Developmental influence on respiratory epithelia to Paramyxoviridae infection, beta-defensin expression and adenoviral delivery of a beta-defensin gene

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Developmental influence on respiratory epithelia to Paramyxoviridae infection, beta-defensin expression and adenoviral delivery of a beta-defensin gene

by

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CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation describes the developmental influence of Paramyxoviridae infection, beta-defensin expression, and adenoviral mediated gene therapy on respiratory epithelia in the perinatal lamb model. 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 briefly gives details of the dissertation organization along with a general review of human Paramyxoviral infection, beta-defensins, adenoviral-mediated gene therapy and their relationship to respiratory epithelia. This is followed by a review of lambs as models for Paramyxoviral infection, developmental diseases of the respiratory system, beta-defensin expression and adenoviral mediated gene therapy. Chapter 1 concludes with the specific aims of the dissertation. Chapter 2 characterizes the clinicopathological features of the respiratory syncytial virus infection in the preterm lamb model and demonstrates age-dependent enhancement of infection. Chapter 3 defines the developmental tissue distribution mRNA expression and peptide of sheep beta-defensin-2 during fetal to adult development. Chapter 4 describes the clinicopathological effects of adenoviral mediated gene therapy of a beta-defensin on concurrent Paramyxoviral infection. Chapter 5 is in the form of a general conclusion and ends with suggestions for future study from this work.
Overview

Preterm infants are at increased risk for severe Paramyxoviral infection and respiratory disease can result in death (14, 158). Deficient innate immunity, in part, may predispose preterm infants to infection, since the respiratory epithelia is immature and many innate antimicrobial peptides/proteins are developmentally regulated (63, 105, 206). Suboptimal expression of developmentally-regulated beta-defensins by epithelia in the preterm infant may sufficiently alter pulmonary innate immunity to increase the susceptibility of respiratory epithelia for severe Paramyxoviral infection. Furthermore, supplemental expression of a beta-defensin using gene therapy could enhance the innate antiviral immunity of the pulmonary antimicrobial milieu to minimize susceptibility to severe Paramyxoviral infection.

Adenoviral-mediated gene therapy has been used in pulmonary research and it has a predilection of respiratory epithelia (122, 153). The preterm/neonatal lamb was chosen as a potential model since lambs are susceptible to Paramyxoviral infection, express beta-defensins, are known recipients of adenoviral mediated gene therapy and are commonly used to study preterm/perinatal respiratory disease. This work 1) characterizes RSV infection in the preterm lamb model, 2) defines the developmental distribution and expression of sheep beta-defensin-2 (SBD2) and 3) describes the influence human beta-defensin-6 (HBD6) expression by adenoviral mediated gene therapy on the clinicopathological features of PIV3 infection.
Paramyxoviridae

The Paramyxoviridae family of viruses is an important source of disease in human and veterinary medicine. Classic Paramyxoviridae diseases such as measles or rinderpest are feared not only out of respect for their devastation on susceptible populations, but in recent times have become prominent in their potential use as biological agents in terrorist-like activity (29, 77). Ubiquitous Paramyxoviridae, such as respiratory syncytial viruses or parainfluenza viruses, cause widespread seasonal morbidity and, in high risk populations, can cause severe disease requiring hospitalization with increased risk for mortality (68). Recently, concerns over the impact of emerging Paramyxoviridae such as human metapneumovirus or the zoonotic Nipah and Hendra viruses further exemplify the clinical diversity and significance of this taxonomic group in human and veterinary medicine (19, 183).

Common features of the Paramyxoviridae family include a spherical to pleomorphic virion greater than 150nm (88). Virions are composed of an outer lipid envelope derived from the host cell's plasma membrane during budding (i.e. exocytosis from the cell). The envelope typically contains 2-3 viral encoded transmembrane glycoproteins that form projections (peplomers) on the envelope surface and are visible by electron microscopy. The envelope surrounds a nucleocapsid composed of a single molecule of single stranded, negative sense RNA with viral matrix proteins and a viral-encoded polymerase. Virions are readily susceptible to inactivation by heat, lipid solvents, ionic and nonionic detergents, formaldehyde, ultraviolet light and oxidizing agents. The susceptibility of the virions
to detergent action is consistent with the concept of hand washing as a significant means to prevent RSV transmission (90).

The fundamentals of Paramyxoviridae replication are similar among family members with minor differences in proteins used for specific roles (e.g. attachment). Attachment and fusion of the virion to the host cell is mediated by specific transmembrane protein(s). Upon cellular entry, the virion-associated polymerase begins to make positive sense mRNA (from the virion’s negative sense single strand RNA) for subsequent translation by the host cell's translation machinery. The protein products include those for synthesis of progeny negative-sense ssRNA and for structural use in final virion assembly. Replication of Paramyxoviridae is dependent on the presence of viral polymerase and host cell translation machinery, therefore the naked viral RNA is not by itself infectious (88, 144).

The Paramyxoviridae family is sub-classified by numerous morphologic and genetic features into two subfamilies: the Paramyxovirinae and Pneumovirinae (Table 1). The Paramyxovirinae subfamily is composed of four genera: Respiroviruses, Rubulaviruses, Morbilliviruses and the recently proposed Henipaviruses (19). The Pneumovirinae subfamily is composed of two genera: Pneumoviruses and Metapneumoviruses (88, 183). For this dissertation, the viruses of interest are respiratory syncytial virus (RSV) and parainfluenza virus-3 (PIV3) species. These viral diseases will be characterized in the following pages.
Classification and structure

Respiratory Syncytial Virus (RSV)

The Pneumovirus genus is composed of three fundamental species: human RSV (hRSV), bovine RSV (bRSV) and murine pneumonia virus (33). Ovine and caprine strains of RSV have been described but there is a lack of consensus for their designation as either a subgroup (strain) of bRSV or as distinct separate species (59, 88, 202).

The RSV genome is comprised of ten open reading frames that correspond to eleven viral proteins products. The specific proteins with their respective size and putative function are summarized in Table 2. Two of the major proteins in RSV biology are the attachment (G) and fusion (F) proteins and these transmembrane glycoproteins are major targets for neutralizing antibodies (201).

The G protein plays a major role in the attachment of the RSV as demonstrated in vitro by anti-G protein antibodies blocking attachment of virions to HeLa cells (106). However, the G protein is not essential for viral propagation and assembly as deletion of the G protein diminishes, but does not abolish, the capacity for replication (33). This suggests an alternative mechanism for cellular entry. The G protein is highly glycosylated which may function to mediate the binding of G protein to cells for entry. Removal of RSV virion N- and O-linked oligosaccharides by glycanases resulted in a 97% reduction in infectivity (99). Furthermore, RSV binding is minimal to CHO cells that are glycosylation- and glycosaminoglycans (GAG)-deficient, suggesting that surface glycosyl groups and GAGs participate in RSV
binding (119). Recent *in vitro* work on Hep2 cells using an L-selectin antagonist (TCB1269) prevented RSV binding and suggested L-selectin-like moieties may be involved in RSV binding. Further efforts by that group of investigators identified annexin II, a plasminogen- and calcium-binding protein, as a binding agent to G protein and a putative RSV receptor (118). The extent of G protein glycosylation is variable between cell lines. This variable glycosylation, which likely occurs *in vivo*, in combination with a relatively low (44%) conservation in amino acid sequence are believed to provide a significant portion of the antigenic diversity among strains (33). While the G protein is classically described as the major mediator for RSV cellular attachment, it is evident that additional factors to the G protein are involved and further work is needed to fully define their significance.

The second major RSV protein is the F protein. Compared to the G protein, the F protein is more highly conserved among the strains of RSV and thus makes for a preferential target in RSV immunoprophylaxis (201). The F protein mediates fusion (or penetration) by the enveloped virion to the cell plasma membrane and provides cellular entry (33). Binding of host defense factors may modulate the F protein's ability for fusion and ensuing viral activity. For example, recent *in vitro* work suggests surfactant protein A binding to the F protein enhances viral replication and host cell IL-8 expression while F protein binding by lactoferrin inhibits viral replication and IL-8 expression (159). Another product of the F protein is the fusion of infected to adjacent cells for formation of the characteristic multinucleated "syncytia". Syncytial cell formation is mediated in part by the three N-glycan chains of the F protein and most of the fusion activity is attributed to the N500-glycan subunit (207). Once in the
cell, interaction of the F protein with Rho A, a cellular GTPase involved with actin reorganization, facilitates syncytial formation (143). Furthermore, RSV cellular infection promotes cytokeratin 17 expression by the host cell via the NF-κB signaling pathway and this protein is localized primarily to cells forming syncytia (44). RSV fusion activity and infection may be enhanced by neuraminidase activity to remove the sialic acid residues on the virion membrane (13). Neuraminidase activity is not a feature of RSV translational products and this opens the door to an interesting question concerning potential polymicrobial disease. Would coinfection with RSV and neuraminidase-producing viruses (e.g. influenza or parainfluenza virus), bacteria (e.g. Pseudomonas or Streptococcus) and fungi (e.g. Cryptococcus or Pneumocystis) lead to enhanced RSV disease (13)? Seemingly there is little evidence of coinfection with RSV as being clinically relevant, even with secondary bacterial involvement (126).

**Parainfluenza virus-3 (PIV3)**

Parainfluenza virus-3 belongs to the Respirovirus genus. Representative species in this genus include bovine PIV3, human PIV3, human PIV1, Sendai virus (murine PIV1) and Simian virus-10. Some ovine isolates of bovine PIV3, a host restricted ruminant virus, are serologically distinct suggesting differences, but viral taxonomy of these ovine strains thus far remain classified as bovine PIV3 (3, 88).

The human PIV3 genome comprises six different gene products and their respective functions are listed in Table 3. Similar to RSV, PIV3 has two major
transmembrane glycoproteins that are targets for neutralizing antibodies in host defense, the hemagglutinin neuraminidase (HN) and fusion (F) proteins (68).

The HN transmembrane glycoprotein is comparable to the G protein of RSV in the important function of viral attachment to host cells. For initial interaction, viral particles bind to sialic acid-containing receptors on the host cells surface via the HN moiety (75). The HN protein then may enhance the fusion function of the F protein for increased efficacy of host cell and virion fusion (150). Following viral replication and virion packaging, the neuraminidase function of the HN protein cleaves virion-bound attachments allowing infection of additional cells. (68, 150).

The PIV3 fusion (F) transmembrane glycoprotein is also analogous in function to the RSV F protein as it is important in fusion of the virion with host cells (75). Syncytial cell formation can be seen during PIV3 infection but the frequency tends to be less numerous than that of RSV infection and this may be due to the dependence of F protein on the presence of homotypic HN protein for efficient function (49, 150). An additional function described for the PIV3 F protein is hemolysis activity but its role, if any, in PIV3 pathogenesis is not well characterized (49, 75)

Pathogenesis and lesions

RSV

Transmission of the RSV virus from an infected individual is primarily through infected droplets on hands and fomites, or less likely in large aerosol. These infected particles then enter the nose or conjunctiva of a susceptible individual (69).
Replication of RSV in the nasopharyngeal epithelium is followed 1-3 days later by spread to the epithelium of the lower respiratory tract. Necrotizing bronchiolitis with cellular necrosis and sloughing, catarrhal exudate and neutrophilic infiltrates in the airway lumen leads to partial or complete occlusion of the small airways, these result in the characteristic clinical signs of wheezing, atelectasis and hyperinflation. Interstitial pneumonia with mononuclear cell (macrophages, lymphocytes, etc.) infiltrate is also seen and may represent a latter stage of infection (68). The current view of RSV disease in humans is it lacks secondary bacterial pulmonary involvement, but interestingly bacterial urinary tract infections are associated with severe RSV disease (126, 140, 181). This is in contrast to ruminant RSV or PIV3 infection, which is thought predispose the animal to secondary bacterial pneumonia (3).

**PIV3**

The pathogenesis of PIV3 is similar in to that of RSV. PIV3 is transmitted by aerosol or direct contact of infected secretions. The PIV3 virus has a propensity to replicate in the upper airway epithelium. The necrosis of the infected airway cells can lead to secondary bacterial infection, most commonly in the trachea. In a small proportion of individuals (especially seen in high risk groups such as the very young or immunocompromised) the PIV3 infection can go to the epithelium of the lower respiratory tract and cause necrotizing bronchiolitis and interstitial pneumonia leading to hospitalization (155, 194).
Clinical features

RSV

RSV infection is a globally important respiratory disease of infants and children. In the United States alone RSV infection is the most common cause of respiratory disease leading to hospitalization in children costing an estimated $300 to $400 million annually in the late 1980's (68, 81). RSV is the most important cause of bronchiolitis in children and the incidence of bronchiolitis hospitalization in children under 1 year of age increased 2.4 fold from 1980 to 1996 (67, 169). Infection is ubiquitous and most children have been infected by 2 years of age (62). Surveillance of RSV infection suggests that seasonal outbreaks typically peak in the mid winter months of January to February with minimal endemic activity during the rest of the year (68). In most people, RSV infection leads to upper respiratory infection that is similar to or somewhat worse than a typical cold. However, in specific populations RSV infection can lead to more severe manifestations such as croup, apnea, bronchiolitis, or pneumonia with an increased risk of hospitalization and mortality. High-risk populations include preterm, young or low birth-weight infants, immunodeficient individuals, those with cardiac or pulmonary disease and elderly adults (6, 186). A putative sequela of severe RSV infection is reactive airway disease and asthma (147). This association is controversial and some have proposed that instead of RSV predisposing to reactive airway disease and asthma, that severe RSV infection may act as a marker for those predisposed to these diseases (152).
There are at least two major strains of hRSV, A and B, with distinct antigenic differences present in the G protein (128). Within each strain are various subtypes (genotypes). RSV outbreaks are usually characterized by a single strain and subtype with subsequent infections by another strain or subtype. This is thought to partially explain the apparent susceptibility of recently infected individuals to subsequent RSV infection (67).

PIV3

PIV3 is an important and ubiquitous cause of seasonal respiratory disease typically manifesting in the late spring to early summer (68). Infection in a healthy individual is localized primarily to the upper respiratory tract and is often self-limiting with coryza and cough as the most common presentation (68, 144, 155). However, PIV3 can cause more serious respiratory disease such as severe laryngotracheitis ("croupy" cough), bronchiolitis and pneumonia leading to hospitalization and on rare occasions, death (75). Risk factors for severe manifestations of parainfluenza virus infection include: primarily lung disease (e.g. cystic fibrosis or asthma), the immunocompromised (e.g. transplant recipients), young children, and the elderly (57, 68, 76). In young children, PIV3 infection is more frequent than other parainfluenza viruses with about 15% of PIV3 infections involving the lower respiratory tract and of these 0.3% require hospitalization amounting to nearly 20,000 children hospitalized annually (41, 61) Parainfluenza viruses, along with other viruses are increasingly recognized in exacerbation of cystic fibrosis lung disease and may predispose to secondary bacterial infection, including
Pseudomonas aeruginosa which is a persistent problem in cystic fibrosis lungs (189). Young children and infants with cystic fibrosis are at increased risk for lower respiratory tract infections by PIV3 and other community-acquired viruses resulting in increased hospitalization rates and chronic lung damage (76, 188).

Treatment and Prevention

RSV

Currently, there is no efficacious treatment for severe RSV and in most cases supportive treatment includes: bronchodilators, corticosteroids, fluid therapy, antiviral agents (e.g. ribavirin) and oxygen management (142). Recent developments include a humanized monoclonal antibody (Palivizumab) against the F protein, which can prevent severe manifestations of RSV disease that require hospitalization, but it does not prevent infection. However, because of the high costs of individual treatment the use of Palivizumab is often restricted to high-risk patients (173). A recent concern is that RSV mutants have been selected in vitro that resist Palivizumab prophylaxis in vivo, suggesting that this therapy may select for future resistant strains of virus analogous to antibiotic therapy in bacteria (205). The use of Palivizumab to prevent severe RSV disease is functionally related to the natural disease setting where protection from severe RSV disease in the elderly was correlated with neutralizing antibody to RSV (186).

An obvious solution is to make a vaccine against the F protein (or perhaps the G protein) for use in the general and high risk populations. This could greatly reduce
RSV-associated health costs, increase worker productivity during seasonal outbreaks and minimize severe RSV infection in high-risk groups. Unfortunately, vaccine development has been slow and cautious since the 1960's in which formalin-inactivated RSV vaccines were clinically tested and some of the vaccinates developed severe RSV disease following natural exposure (173). While the vaccinates had high levels of non-neutralizing and non protective antibodies, it is not clear to what extent, if any, the process of formalin inactivation had on the lack of neutralizing antibodies (149). The mechanisms responsible for this are ill-defined and have contributed to increased research using modified-live or subunit RSV vaccines for various subpopulations based on risk and benefit. Modified live vaccines are being directed for use in young infants and perhaps the elderly while subunit vaccines are being targeted more for high-risk RSV-seropositive children, pregnant women and the elderly (149). Some of the modified live-vaccine research utilizes as the core virus bovine PIV3, which is attenuated to non pathogenic in humans (47). Interestingly, a bovine PIV3 chimeric vaccine expressing human RSV G and F proteins offered protection to challenge against human RSV and PIV3 in hamsters (70). While there have been multiple RSV vaccine trials in the past decade, not a single one is licensed for general use and attempts at future government approval may be rigorous in light of previous RSV vaccine problems.

PIV3

Treatment for PIV3 infection is usually symptomatic, but more severe manifestations such as respiratory compromise due to severe laryngotracheitis
require nebulized bronchodilators to increase airway diameter and corticosteroids to decrease inflammation. Exposure to humidified or cold air has anecdotally been described to alleviate PIV3-associated mucosal edema and to thin secretions, but this has not been thoroughly evaluated scientifically. Ribavirin, a guanine analog, has activity against PIV3, but is often administered too late in the infection process for significant efficacy. It may be of more value for persistent infection sometimes seen in immunocompromised individuals (194).

Currently there is no licensed vaccine for PIV3 in humans. Work on PIV3 vaccines (both subunit and modified live vaccines) are progressing with one of the investigated modified live vaccines strains being bovine PIV3 (194). The bovine PIV3 strain is early in development but a modified-live human PIV3 vaccine candidate that has promising efficacy will soon be in phase III human trials (47).

Summary

Paramyxoviridae such as RSV and PIV3 are important epitheliotropic respiratory pathogens, especially for high-risk patients such as preterm infants and the elderly. Preterm infants have increased susceptibility to develop severe manifestations of RSV and PIV3 infection. Specific prophylactic and treatment regimes to RSV and PIV3 are deficient, in part, due to lack of a good animal model for study. Furthermore, an animal model that demonstrates age-dependent disease similar to preterm infants is lacking. The preterm lamb would be an excellent candidate for a Paramyxoviral infection animal model.
Beta-Defensins

Antimicrobial Peptide/Proteins

The body's mucosal surfaces such as the respiratory tract are designed with an overlapping complex array of immune defense factors that protect the host from a constant barrage of exogenous pathogens and disease. One component of this innate defense milieu is antimicrobial peptides/proteins. These peptides and proteins are increasingly appreciated for relatively inconspicuous, but active role. It is amazing an observation of antibacterial activity was made so many years ago. Early recognition of this function was made in the early twentieth century when Dr. Alexander Fleming made an insightful discovery that respiratory secretions had antibacterial activity and he characterized the properties of the product he termed "lysozyme" (51, 52). In the past few decades discoveries in the field have highlighted how antimicrobial peptide/proteins are ubiquitous in nature. For instance, the diversity of the small cationic peptides called "defensins" are evident in their taxonomical range from insects (100) to amphibians (204) to mammals (56, 167) and in their cellular localization from leukocytes (168) to epithelia (42). In recent years with the advances in molecular techniques and computational searches, novel genes involved in this defense are continually being discovered (166).

Overview of pulmonary defense

In the respiratory tract reside multiple levels of anatomic, cellular and molecular components that work together for, in part, the purpose of innate host defense. A brief overview of this innate system will help establish a foundation for
understanding the roles of antimicrobial peptides. The anatomical arrangement of
the upper respiratory system (e.g. nasal concha) allows for significant particle
deposition, humidification and warmth of inhaled air for presentation to the abundant
gas-exchange regions in alveoli. In much of respiratory tract (primarily nasal cavity,
trachea, bronchi, and bronchioles) ciliated epithelial cells function to remove cellular
and foreign particulate debris by way of the mucociliary blanket. Alveoli, which lack a
functional ciliary system, have residential alveolar macrophages that are responsible
"cleaning" up any particulate matter and cellular debris (117). These anatomic
structures and specialized cells contribute to the homeostasis and health of the
respiratory tract.

Cellular secretions of the respiratory tract provide molecules that participate in
host defense including mucus (111), surfactant protein A and D (105), lysozyme
(52), transferrin (192), ficolins (1, 120) lactoferrin (180), secretory leukocyte
proteinase inhibitor (156), alpha-1 antitrypsin (27), secretory IgA (146),
phospholipase A2 (45), bactericidal permeability inducing protein (BPI, 55),
cathelicidin (10), anionic peptide (72) and defensins (174) to name a few. While the
pulmonary system has multiple protective mechanisms for host defense, infectious
respiratory disease is still a ubiquitous problem with new respiratory diseases
continually emerging (127, 132, 170). Alterations in the expression/function of some
of these innate antimicrobial peptides/proteins have been suggested as predisposing
factors to disease. Reduced surfactant A and D in the lung decreases bacterial and
viral clearance and enhances inflammation associated with the disease (105).
Furthermore, inactivation of beta-defensins may predispose cystic fibrosis patients to
chronic lung infection and inflammation (58, 63). Intuitively, the functional loss of just one antimicrobial peptide/protein should not seem to be too biologically significant given the abundant redundancy of the "antimicrobial soup". However, after further examination it appears that the antimicrobial activity of many of these innate molecules is enhanced by the presence of other innate molecules. This suggests that the functional loss of even one type of innate molecule may confer a generalized loss of efficacy to the rest of the "antimicrobial soup" (165). An example of this was seen in mice deficient in mouse beta-defensin-1 function as these mice had delayed pulmonary clearance of inoculated *Haemophilus influenzae* (131). Also, HBD4 when combined with lysozyme or HBD3 has increased antibacterial activity suggesting synergism (58).

Some scientists have taken the functional role of antimicrobial peptides/proteins to the "next step" by focusing on therapeutic intervention of disease by enhanced antimicrobial peptide/protein expression or by direct supplementation (12, 165). For instance, enhanced cathelicidin (LL-37/hCAP-18) expression has been suggested as a possible therapy for treatment of lung infection in cystic fibrosis patients (12). Also, airway supplementation of SMAP29, a cathelicidin of sheep, reduced *M. haemolytica* numbers in bronchoalveolar lavage and lesions suggesting a potential therapeutic use with minimal pulmonary toxicity (24). These and other studies have encouraged entrepreneurial development as many pharmaceutical companies are in the process of testing antimicrobial peptides/proteins for topical to systemic use (96).
**Defensins**

Defensins are widespread in nature and can be found in many fauna and flora (154). Defensins are a conserved class of antimicrobial peptides characterized by a cationic charge, antimicrobial activity and six to eight highly conserved cysteine residues that form intramolecular disulfide bonds. There are three families of defensins including alpha-, beta- and theta-defensins each characterized by the organization of intramolecular disulfide bonds (200). As seen above, antimicrobial peptides/proteins are important components of respiratory innate defense. Of these many respiratory molecules, much interest has been given to the beta-defensins, in part, because of their epithelial expression, regulation, small size and their decreased expression/function as a putative factor for increased susceptibility to respiratory disease (63, 165). Bovine tracheal antimicrobial peptide (TAP) was the first mammalian beta-defensin characterized in detail and this was followed by a large increase in beta-defensin research (42, 43, 165).

**Beta-defensin - structure**

Beta-defensins have a conserved structure of six-cysteine residues (generally C-X6-C-X4-C-X9-C-X6-C-C) forming C1-C5, C2-C4 and C3-C6 disulfide intramolecular bonds with a molecular structure forming three antiparallel beta-sheets (79, 82, 178). In humans, there are at least six defined beta-defensin gene products classified human beta-defensins (HBD) 1-6 (9, 16, 58, 71, 196). However, a recent computational search suggests there may be well over thirty putative beta-defensin genes and expression of many of these gene products are currently being
characterized thereby adding to the knowledge of human beta-defensins (151, 166). Many of these beta-defensins are located in epithelial cells, but in some species beta-defensins are also found in leukocytes (167, 168). There are some families of peptides and proteins that closely resemble beta-defensins in structure and activity. HE2/EP2 belong to a family of proteins located exclusively in the testis and epididymis that form splice variants during processing that have beta-defensin-like structure and antimicrobial activity (53, 185).

**Beta-defensin expression**

Over thirty human beta-defensin genes have been found in up to five different clusters that are syntenic with mouse beta-defensins suggesting these regions are conserved among species (166). Some of these genes, such as HBD1, appear to be constitutively regulated while others such as HBD2 are inducible by pathogens or microbial products (e.g. lipopolysaccharide), or cellular inflammatory elements such as mitogen-activated protein (MAP) kinase or NF-kappa beta (15, 50, 114, 125, 138). During beta-defensin gene expression, exon transcripts are spliced together to form a primary translational product that is called a pre-pro-peptide (82). At the N terminus is the "pre" portion, a highly conserved signal sequence that is important for endoplasmic intracellular trafficking and is cleaved before cellular exocytosis (130). The "pro" portion is often highly anionic and thought to offset the cationic C terminus and may play a role in intracellular trafficking and folding of the C terminus portion (96). The pro-peptide is transported extracellularly where the pro portion is cleaved to form the active peptide (78). This last point is not as well-defined and
some have suggested only the mature peptide is secreted onto the surface (197). Further work is needed for understanding beta-defensin processing.

**Beta-defensin - antimicrobial activity**

Beta-defensins have a wide spectrum of antimicrobial activity with efficacy against several species of bacteria, fungi, protozoan parasites and viruses (64, 92, 164, 203). The mechanism(s) for this antimicrobial activity is not clearly defined, but a common thought is that there is alteration of the pathogens outer membrane (198). Defensins are cationic and are able to interact with pathogens having a negative surface membrane charge. Increase in the net-negative charge of *Staphylococcus aureus* membranes by *mprF* mutation resulted in increased killing by neutrophil alpha-defensins (145). This interaction by electrostatic forces is the proposed first phase of defensin interaction with a pathogen. The next phase is generally believed to involve integration of the defensin's hydrophobic region with the membrane of the pathogen and there are three putative models for this interaction. The first is the barrel stave model in which defensins form an alpha-helical transmembrane "pores". The aggregate channel model differs slightly from the first in that the defensins aggregate as clusters within the membrane forming transient membrane pores. The carpet model suggests the defensins widely cover and integrate into the membrane effectively causing a detergent-like dissolution (96, 104). Increasing the cationic charge experimentally does not necessarily make defensins more antimicrobial since this altered charge effectively increases electrostatic binding to negatively charged pathogens, but often decreases defensin integration into the membrane.
Alterations of the cationic charge, dimer formation or specific amino acid substitutions between defensins may correlate with the spectrum of antimicrobial activity; however the intramolecular disulfide bonds are not required for antimicrobial activity (161, 195). The epithelial distribution of many human beta-defensins at mucosal surfaces (respiratory, gastrointestinal, urogenital, etc.) and glands make them an important first line of defense against pathogens (198). The antimicrobial activity of beta-defensins is at very small (nanogram to microgram/milliliter) doses, but there is some evidence that at higher doses the host cell cytotoxicity may occur (154). The relative lack of toxicity in host cells is thought to be related to the comparatively lower anionic residues on the cellular membrane (165).

Defensin activity against microbial membranes, while popular in the current literature, may not explain all of the direct antimicrobial functions of beta-defensins. Some have suggested that intracellular effects of defensin expression may be surprisingly important for innate defense against organisms (165). For example, a standard antiviral concept is that defensin activity is effective against only enveloped viruses (i.e. those with outer membranes), however, in an in vitro gene therapy experiment using an adenovirus vector (a non enveloped virus), adenovirus infection was inhibited 3-5 fold by HD5 (an alpha-defensin found in intestinal crypts) and HBD1 expression (37, 64, 87). Additional evidence that antiviral activity may not be due to direct membrane alteration was shown as human alpha-defensin-1 inhibited human immunodeficiency virus (HIV) infection after cellular entry of the virus. The authors of that study discussed the possibility that defensins alter cell signaling pathways to inhibit viral replication (30). While direct effects of defensin mediated
membrane damage is important for antimicrobial activity, additional mechanisms need to be further elucidated.

**Beta defensins - additional roles**

Beta-defensins have an increasing number of functions that are rapidly becoming more clearly defined. In addition to having direct antimicrobial properties, beta-defensins are increasingly appreciated for their suggested roles in innate and adaptive immune responses and in cell regulation (124, 154).

**Chemotaxis**

Recent evidence suggests beta-defensins may function in cellular chemotaxis by interacting with the chemokine receptors. Human beta-defensins-1 &-2 are ligands for CC-chemokine receptor-6 (CCR6), a surface receptor of CD45RO+ memory T cells and bone-marrow derived immature dendritic cells (109, 171, 199). HBD2 has also been shown to function as a chemotactic agent for TNF-alpha primed neutrophils and the purported mechanism was again CCR6 interaction (135). The CCR6 mechanism is not exclusive as HBD2 can also recruit mast cells via a CCR6 independent pathway that likely signals through G protein-phospholipase C (134). Interestingly, the concept of defensins signaling as chemokines has furthered research which recently discovered some chemokines have antimicrobial-like activity (32). As seen in the examples above, beta-defensins can participate in the recruitment of cellular inflammation which is important for the advancement of an immune response.
Immune regulation

Once leukocytes arrive at sites of inflammation defensins may continue to influence the inflammatory response. HBD2, an inducible peptide, can cause mast cell degranulation (136). Mouse beta-defensin-2 (MBD2) can act as a ligand for toll-like receptor-4 on dendritic cells resulting in activation and maturation similar to binding by LPS. These activated dendritic cells expressed T_H1 polarized proinflammatory cytokines such as IL-12, IL1 and IL-6 along with chemokines and increased surface expression of MHCII and CCR7 molecules (17). Beta-defensin binding to an antigen may also regulate the type and magnitude of immune response. In a DNA vaccine model, MBD2 was fused to a nonimmunogenic B-cell lymphoma epitope (sFv38) and immunized mice had a robust humoral response and antitumor immunity (18). Related work showed that the humoral response to (ovalbumin) OVA alone versus OVA bound to HBD1 or HBD2 was significantly different with the bound beta-defensin causing an increase in the OVA-specific IgG levels at twenty one days post immunization (23). A proposed explanation for this augmentation of adaptive immunity could be due to enhanced uptake of the antigen-bound beta-defensin. This along with defensin activation and maturation of the dendric cells via CCR6 complex may augment the response (197).

Epithelial Regulation and Influence of Beta-Defensins

Additional effects of beta-defensins are present in the resolution of wounds and inflammation. The inducible HBD2, but not the constitutive HBD1, had increased mRNA and peptide expression during corneal re-epithelialization suggesting that
HBD2 may function in 1) an antimicrobial role near a site of injury and/or 2) a role modulating cellular proliferation (124). In contrast HBD1 expression is low during proliferation of cells but increased at differentiation. Hyper-expression of HBD1 in cells causes rapid differentiation suggesting HBD1 is a regulator of cellular differentiation (54).

Summary

Beta-defensins are a subclass of antimicrobial peptides that are often found at epithelial surfaces consistent with their putative role as contributing to the first line of defense against environmental pathogens. Reduction in function or expression of just one beta-defensin can greatly affect the innate immunity status of the host. Expression of several beta-defensins by pulmonary epithelia appears to be developmentally regulated. The immature respiratory epithelia of preterm infants may have functionally insufficient beta-defensin expression which predisposes them to severe RSV and PIV3 disease.

Gene Therapy

Our understanding of what constitutes "disease" and disease processes has been progressively defined at the molecular level. Aberrations of normal host gene expression have been increasingly shown to play an associative to causative role in disease pathogenesis. This corresponds to the frequent news reports heralding potential new "heart-attack" to "Alzheimer's disease" genes (74, 93). The basic genomic concepts of disease, from predisposition to causation, lead to the ultimate
questions of how to functionally restore or alter expression for healthy homeostasis. "Gene therapy" can basically be defined as a medical application using molecular techniques to alter disease at the level of genes (98).

Some of the earliest uses of gene therapy directed placement of selected genes into target cells as plasmids. Techniques to transfer nucleotide sequences included electroporation, microinjection and particle-mediated bombardment, or by altering the exterior of nucleotide sequence with calcium ions or cationic lipids to enhance transfers across the plasma membrane (98). While these techniques offer unlimited size of genes that can be transferred, they have severe limitations in the efficiency of cellular uptake, persistence, and gene expression levels. Researchers have since expanded into other methods of gene transfer.

Viruses are by nature excellent vectors for gene therapy in that they routinely perform the "basic" elements desired for effective gene therapy - they are able to enter the cell carrying genes and allow for transcription/translation of the newly introduced genes (179). Viral vectors are often made to carry gene inserts (cassettes) by deleting portions of the wild type-viral genome, usually those regions associated with virulence, replication and/or toxicity (116). The benefits of using viruses for gene therapy are the comparative ease of cellular entry along with the ability of generally sustained gene expression, depending on the virus. However, there are limitations to using viral vectors including restrictions on the size of inserted gene cassettes into the viral genome, immunologic responses to the "foreign" virus and potential for disease caused by the attenuated vector virus. Each viral class has certain advantages and disadvantages with respective tissue
tropisms that must be recognized prior to application. Specific viral groups utilized as virus vectors include: oncoretroviruses (e.g. murine leukemia virus), lentiviruses (e.g. human immunodeficiency virus), adenoviruses (e.g. human adenovirus-5), adeno-associated viruses (e.g. adeno-associated virus-2), and herpes viruses (herpes simplex virus-1) (98, 179).

In pulmonary diseases, there are many opportunities for gene therapy to have a role in therapeutic intervention. Diseases such as cancer, cystic fibrosis, alpha-1-antitrypsin deficiency and respiratory distress syndrome are prominent examples of this potential (60). Cystic fibrosis is a disease due to a primary genetic defect in the function of the cystic fibrosis transmembrane conductor (CFTR) gene product on the apical portion of epithelial (94). Lack of a functional CFTR results in abnormally thick mucus secretions causing disease in the pancreas, liver, respiratory, reproductive and gastrointestinal tracts (157). Patients with lung abnormalities have a thick respiratory secretions and a predisposition to pulmonary infection including viruses and bacteria, most notably persistent Pseudomonas aeruginosa (39, 189). This predisposition to infection of cystic fibrosis patients is believed to be caused by dysfunction of the pulmonary innate immune system, in part, defensin inactivation (11, 12, 63). While most of the gene therapy efforts in cystic fibrosis patients have focused on restoring CFTR expression in pulmonary epithelia, additional efforts to restore pulmonary host defenses have also been explored (12). These have mainly focused on antimicrobial peptide's expression against bacterial infection, whereas beta-defensin gene therapy for enhancing antiviral immunity against Paramyxoviridae infection is a more novel concept.
Adenoviral-mediated gene therapy

A common viral vector used in pulmonary cystic fibrosis research is the adenovirus (122). Part of the appeal of adenovirus vectors is the tropism to epithelia, the cellular site associated with CFTR disease. Adenoviruses typically bind to the coxsackie-adenovirus receptor (CAR) that is located preferentially on the basolateral portion of differentiated epithelia (153). Additionally, the adenovirus may attach to various epithelial adhesion molecules (e.g. alpha_v-beta_3 or alpha_v-beta_5 integrins) for enhanced CAR mediated transfer or bind to phospholipid for epithelial entry during surfactant turnover (8, 191).

Adenoviruses used for gene therapy typically have deleted regions of the genome in order to minimize viral toxicity and allow room for target gene insertions. Some of the early adenoviruses (first generation) had deletions in the E1 and/or E3 regions of the viral genome (5, 116). E1 gene products modulate viral transcription and replication, and thus E1 deletion confers a replication deficient virus (28). Interestingly, both E1 and E3 gene products were recently shown to have immunosuppressive effects, thus E1/E3 deleted vector viruses elicits more of a host immune response than the wild type virus (80, 160). Host reaction to adenoviral vectors include increased IL-8 production in bronchioles along with increased intercellular adhesion molecule-1 (ICAM-1) on epithelia which is consistent with the increased neutrophilic infiltration typically seen following adenovirus vector inoculation (97, 133). Additional deletions in the E2 and E4 regions (second or third generations) or deletion of the entire coding region (helper-dependent gutted Ad vectors) have reduced host immune response with similar transfection efficacy but
variable duration (110, 116). Cellular localization of adenoviral gene expression is often assessed by use of a reporter gene, such as the lacZ gene that encodes for beta-galactosidase (122). Beta-galactosidase exposure to X-gal (4-bromo-5-chloro-3-indoyl-beta-D-galactopyranoside) leaves a blue reaction product in tissues and differentiation from endogenous mammalian beta-galactosidases is easily discerned using slightly alkaline pH (190).

**Summary**

Adenoviruses are common vectors for respiratory gene therapy, in part, because of their natural epitheliotropic nature. Adenoviral delivery of antimicrobial peptides has been attempted for eliminating persistent bacterial infection but little is known concerning viral infection. Adenoviral delivery of a beta-defensin gene could enhance the innate pulmonary immunity and reduce the severity of Paramyxoviral infection.

**The Lamb Model**

Currently, there is no well-recognized model for the study of the severe RSV or PIV3 disease associated with preterm and young neonatal infants. Paramyxoviridae infection research in high risk groups such as preterm infants is limited by technical difficulty, practical and ethical concerns (163, 184). Study of Paramyxoviridae infection in animal models requires comparable clinical disease, lesion development, immunity, or other features that relate to the specific aims of the experiment (73).
RSV animal models

Animal models for study of human RSV (hRSV) pathogenesis and immune response include rats (36), mice (139), ferrets (31), guinea pigs (21), hamsters (26), lambs (112) and nonhuman primates (121). Infection in the chimpanzee is viewed by some to be the most analogous to human disease, however, animal availability is limited, few are naive and they require specialized facilities and management making this model impractical (33). Mice are often used in models for human disease (including RSV) because of the ease of handling and availability of strains with specific knockout genes (139). However, hRSV infection in rodents produces only mild bronchitis without overt respiratory disease making distinctions about severe infection difficult. Related Pneumovirus models include mouse pneumonia virus (MPV) in mice and bovine respiratory syncytial virus (BRSV) infection of ruminants. MPV does not cause significant disease or lesions in mice and so is not a viable model. BRSV is genomically, antigenically and functionally related to human RSV and is host-adapted for ruminant infection causing significant clinical pulmonary disease and lesions compatible with human RSV infection (33, 102, 202). BRSV infection in ruminants is recognized as a parallel viral model for human RSV infection (73).

PIV3 animal models

Animal models for PIV3 infection are not as aggressively pursued as RSV in part due to the less severe nature of PIV3 infection in humans and due to some of the "overlap" from the RSV research. Animals used for human PIV3 infection
research are similar to RSV models and include rats (141), guinea pigs (38), mice (182), hamsters (48), and nonhuman primates (46). Analogous viral models for PIV3 include the bovine PIV3 which infects ruminants (66, 103, 176) and the Sendai virus of mice and rats (175, 187). Recently a caviid parainfluenza virus-3 was described in guinea pigs; however, clinical signs and lesions were minimal and it does not appear to be an applicable model of human PIV3 disease (172). The ovine model of (bovine) PIV3 disease results in consistent lesions and is well-recognized (66, 103).

**Comparative pulmonary development**

Preterm infants are prone to respiratory distress syndrome and this is a direct result of immature respiratory epithelial development and surfactant synthesis (95, 129). As discussed later in this chapter, pulmonary epithelial development nears functional maturity based, in part, on the production of surfactant for lung compliance and the ability to breath outside the womb. The corresponding anatomical growth continues postnatally with maturation of the alveolar stage of development (2, 40). However, preterm and young infants that express inflammatory mediators arising from common perinatal treatment regimes (mechanical ventilation or oxygen therapy) or infection are prone to develop bronchopulmonary dysplasia (BPD, a.k.a. chronic lung disease of immaturity) characterized by primarily by alveolar hypoplasia with variable fibrosis, smooth muscle hyperplasia and vascular alterations (91).

Along with surfactant, many antimicrobial peptides/proteins appear to be developmentally regulated. Neonatal infants have "immature" expression of developmentally regulated bactericidal/permeability-increasing protein (BPI) and
thus are prone to infection (107, 108). Expression of HBD1, an epithelium derived human beta-defensin, is developmentally regulated in the lungs (123). Previous work on the bovine beta-defensin (tracheal antimicrobial peptide - TAP) showed no mRNA expression in 4 or 6 month old fetuses while adult animals had abundant peptide suggesting developmental regulation (42, 43). In the preterm infant, pulmonary antimicrobial peptides/proteins such as beta-defensins may have immature expression which could be a risk factor in development of severe Paramyxoviral infection.

With a lack of consensus for a Paramyxoviral model, numerous animal models are utilized for Paramyxoviral infections each with their own advantages and disadvantages (26). The lamb model has many appealing features that make them an attractive model for use. Moreover, late term fetal and perinatal lambs have proven valuable as pulmonary models for the study of surfactant expression and regulation (177), ventilation-induced injury (86), congenital diaphragmatic hernia (113), chorioamnionitis (7), sleep apnea (112), lung development (40), perinatal pulmonary function (35), ontogeny of pulmonary gene expression (22) and persistent pulmonary hypertension of the newborn (65).

Another consideration for animal models of pulmonary disease is that of lung development. There are progressive classifications to the stages of anatomical development of the lung: embryonic, pseudoglandular, canalicular, saccular and alveolar. In humans (gestation = 40 weeks), the alveolar stage of development has typically started by 36 weeks of gestation (as early as 30 weeks of gestation) and development continues into the postnatal period (101). In sheep (gestation = ~148
days), alveolar development begins around day 120. At birth, lambs are further along in pulmonary development than infants, but both lambs and infants complete alveolar development postnatally (2, 40). The beginning of alveolar development in utero corresponds to the appearance and progressive increase of surfactant apoproteins and phospholipids in the lung (95, 129). Conversely, rodents (term ~19.5 days) are born with pulmonary development still in the saccular stage and true alveolar development begins postnatally (4, 25). Mice delivered even 24 hours before term often do not survive and this corresponds to initial surfactant expression, which occurs just prior to birth and continues to increase postnatally for many days (34, 193). Rodents are a common model in many Paramyxoviridae research labs and are valuable tools for study of RSV immunity at the gene level. However, as indicated, in many ways the anatomical and physiological development of the human lung is more comparable to that of sheep. With the rate of preterm and low birthweight babies on the rise, the ovine model could provide novel insights for the increased severity of Paramyxoviridae infection in developing preterm and neonatal infants (89).

**Sheep beta-defensins**

Two sheep beta-defensins have recently been identified, sheep beta-defensin-1 and sheep beta-defensin-2 (SBD1 and SBD2, respectively) (83). Original efforts described the beta-defensin genes to chromosome 24, but subsequent efforts suggests they reside on chromosome 26 which is more equivalent to bovine chromosome 27 (84, 85). Bovine chromosome 27 has syntenic loci comparable with
human chromosome 8, the location of several human alpha- and beta-defensins
genes (115, 137). Very little is known about SBD1 and SBD2 and much of the
expected localization and functional roles come from extrapolation of other beta-
defensins. A study of beta-defensin expression (SBD1 and SBD2) in the ovine
gastrointestinal tract suggested SBD1 was present in all tissues from tongue to
colon but not in ileum (83). SBD1 mRNA was also present in the trachea, while
SBD2 mRNA was localized only to the small intestine and colon. Also, SBD1 and
SBD2 mRNA expression was markedly variable between animals and
developmental regulation of expression was suggested. SBD2 is not well-
characterized, but sheep SBD2 expression may be a model for innate immunity
during pulmonary disease.

Adenovirus gene therapy

Adenovirus vectors have been used for pulmonary gene therapy in part
because of the tropism for respiratory epithelia (153). Young lambs have been
successfully given adenovirus vectors (e.g. human adenovirus-5) for use as a model
of gene transfer in cystic fibrosis patients (122, 148). The use of adenovirus vectors
in the lambs of this dissertation were partially modeled after these studies.

Summary

The lamb is a proven model for late term fetal and perinatal pulmonary
disease research. Lambs are naturally susceptible to bovine RSV an analogous viral
model to human RSV infection. Beta-defensins have been identified in sheep, but
have not been thoroughly characterized concerning expression and distribution
during development. Additionally, adenoviral transfection has been successfully
performed in young lambs. The preterm/neonatal lamb is an excellent candidate
model for study of developmental influence on respiratory epithelia to
Paramyxoviridae infection, beta-defensin expression and adenoviral delivery of a
beta-defensin gene.

Summary

Death in the preterm infant is often associated with respiratory disease and
this same age-group is at increased risk for severe Paramyxoviral disease (14, 158).
In high risk patients, innate immunity may play a critical role in their susceptibility to
severe Paramyxoviridae infection (158, 206). Developmental regulation of
pulmonary antimicrobial peptides/proteins such as beta-defensins may play a role in
this susceptibility. Developmental distribution and expression of SBD2 is not known,
but developmental regulation of expression has been suggested for the
gastrointestinal tract (83). Augmentation of beta-defensin expression by gene
therapy may provide an elevated innate immune status that would alter severity of
Paramyxoviridae infection. The preterm/perinatal ovine model is appealing for study
of developmental expression of beta-defensins as well as Paramyxoviridae infection
and adenoviral gene therapy.

The specific aims of this dissertation were to:

1. Characterize the clinicopathological features of RSV infection in
preterm lambs

2. Define the mRNA expression and peptide distribution of sheep beta-defensin-2 during development.

3. Characterize adenoviral-mediated beta-defensin expression on the clinicopathological features of concurrent parainfluenza virus-3 infection in neonatal lambs.
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Table 1. Representative members of the *Paramyxoviridae* subfamilies (19, 20, 88, 175, 183).

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paramyxovirinae</em></td>
<td>Respirovirus</td>
<td><em>Bovine parainfluenza virus</em> 3; <em>Human parainfluenza viruses</em> 1 &amp; 3; <em>Sendai virus (Murine parainfluenza virus)</em> 1; and <em>Simian virus</em> 10</td>
</tr>
<tr>
<td>Rubulavirus</td>
<td></td>
<td><em>Avian paramyxoviruses</em> 2-9; <em>Human parainfluenza viruses</em> 2 &amp; 4; <em>Menangle virus</em>; <em>Newcastle disease virus (avian parainfluenza virus)</em> 1; <em>Mapeura virus</em>; <em>Mumps virus</em>; <em>Porcine rubulavirus (La Piedad-Michoacan-Mexico virus)</em>; and <em>Simian viruses</em> 5 &amp; 41</td>
</tr>
<tr>
<td>Morbillivirus</td>
<td></td>
<td><em>Measles virus (Edmonston virus)</em>; <em>Canine distemper virus</em>; <em>Rinderpest virus</em>; <em>Phocine distemper virus (Seal distemper virus)</em>; <em>Cetacean morbillivirus virus</em>; and <em>Pest-des-pests-ruminants virus</em></td>
</tr>
<tr>
<td>Henipavirus</td>
<td></td>
<td><em>Hendra virus and Nipah virus</em></td>
</tr>
<tr>
<td>Pneumovirinae</td>
<td>Pneumovirus</td>
<td><em>Bovine respiratory syncytial virus</em>; <em>Human respiratory syncytial virus and Murine pneumonia virus</em></td>
</tr>
<tr>
<td>Metapneumovirus</td>
<td></td>
<td><em>Human Metapneumovirus and Turkey rhinotracheitis virus</em></td>
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### Table 2. Human RSV proteins, weight and putative function (33, 128, 162)

<table>
<thead>
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<th>Molecular weight (kDa)</th>
<th>Putative function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS1</td>
<td>14</td>
<td>Non structural protein; host interferon antagonist?</td>
</tr>
<tr>
<td>NS2</td>
<td>15</td>
<td>Non structural protein; host interferon antagonist?</td>
</tr>
<tr>
<td>N</td>
<td>43</td>
<td>Major nucleocapsid protein; binds viral RNA</td>
</tr>
<tr>
<td>P</td>
<td>27</td>
<td>Nucleocapsid phosphoprotein; chaperone and polymerase cofactor</td>
</tr>
<tr>
<td>M</td>
<td>28</td>
<td>Matrix protein; virion assembly</td>
</tr>
<tr>
<td>SH</td>
<td>7</td>
<td>Small hydrophobic transmembrane protein; function undefined</td>
</tr>
<tr>
<td>G</td>
<td>32</td>
<td>Transmembrane attachment glycoprotein; neutralization and protective antigen</td>
</tr>
<tr>
<td>F</td>
<td>63</td>
<td>Transmembrane fusion glycoprotein; virus penetration and syncytia formation; neutralization and protective antigen</td>
</tr>
<tr>
<td>M2-1</td>
<td>22</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>M2-2</td>
<td>10</td>
<td>Regulatory protein; down regulates transcription and up regulates RNA replication</td>
</tr>
<tr>
<td>L</td>
<td>250</td>
<td>Polymerase</td>
</tr>
</tbody>
</table>

### Table 3. Human PIV3 proteins, weight and putative function (68)

<table>
<thead>
<tr>
<th>PIV3 protein</th>
<th>Molecular weight (kDa)</th>
<th>Putative function(s)</th>
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<tbody>
<tr>
<td>N</td>
<td>58</td>
<td>Major nucleocapsid protein; binds viral RNA</td>
</tr>
<tr>
<td>P</td>
<td>60</td>
<td>Nucleocapsid phosphoprotein; chaperone and polymerase cofactor</td>
</tr>
<tr>
<td>M</td>
<td>40</td>
<td>Matrix protein; virion assembly</td>
</tr>
<tr>
<td>HN</td>
<td>69</td>
<td>Viral attachment and release; major protective antigen</td>
</tr>
<tr>
<td>F</td>
<td>60</td>
<td>Transmembrane fusion glycoprotein; virus penetration and syncytia formation; neutralization and protective antigen</td>
</tr>
<tr>
<td>L</td>
<td>250</td>
<td>Polymerase</td>
</tr>
</tbody>
</table>
CHAPTER 2. REDUCED CLEARANCE OF RESPIRATORY SyncYTIAL VIRUS
INFECTION IN A PRETERM LAMB MODEL

A paper submitted to Microbes and Infection

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Lehmkuhl, Jack M. Gallup, Mark R. Ackermann

Abstract

Respiratory syncytial virus (RSV) causes significant respiratory disease in
children worldwide. For the study of severe RSV disease seen in preterm infants, a
suitable animal model is lacking. The novel hypothesis of this study was that preterm
lambs are susceptible to bovine RSV infection, an analogous pneumovirus with
ruminant host-specificity, and that there would be age-dependent differences in
select RSV disease parameters. During RSV infection, preterm lambs had elevated
temperatures and respiration rates with mild anorexia and cough compared to
controls. Gross lesions included multifocal consolidation and atelectasis with foci of
hyperinflation. Microscopic lesions included multifocal alveolar septal thickening and
bronchiolitis. Immunohistochemistry localized the RSV antigen to all layers of
bronchiolar epithelium from a few basal cells to numerous sloughing epithelia. A few
mononuclear cells were also immunoreactive. To assess for age-dependent
differences in RSV infection, neonatal lambs were infected similarly to the preterm
lambs or with a high-titer viral inoculum. Using morphometry at day 7 of infection,
preterm lambs had significantly more cellular immunoreactivity for RSV antigen (p<0.05) and syncytial cell formation (p<0.05) than either group of neonatal lambs. This work suggests perinatal RSV clearance is age-dependent, which may explain the severity of RSV infection in preterm infants. The preterm lamb model is useful for assessing age-dependent mechanisms of severe RSV infection.

1. Introduction

Respiratory syncytial virus (RSV) infection is a globally important respiratory disease of infants and children. In the United States alone RSV infection is the most common cause of respiratory disease leading to hospitalization in children costing an estimated $300 to $400 million annually [1, 2]. RSV is the major cause of bronchiolitis in children and the incidence of bronchiolitis hospitalization in children under 1 year of age increased 2.4 fold from 1980 to 1996 [3, 4]. Infection is ubiquitous and most children have been infected by 2 years of age [5]. Currently, there is no efficacious treatment for severe RSV infection and supportive treatment during hospitalization includes: bronchodilators, fluid therapy, and oxygen management with more severe cases receiving corticosteroids or antiviral agents (e.g. ribavirin) [6]. Vaccine development has been slow and cautious following a 1960's vaccine trial in which some vaccinates developed severe disease following natural RSV exposure [7]. Recent developments include a humanized monoclonal antibody (Palivizumab) against the fusion (F) protein which can prevent severe manifestations of RSV disease that require hospitalization, but it does not prevent infection. Because of the high costs of individual treatment the use of Palivizumab is
often restricted to high risk patients. Patients with increased risk for hospitalization and mortality due to severe RSV disease include preterm and young neonatal infants [3, 7, 8].

Currently, there is no well-recognized model for the study of the severe RSV disease associated with preterm and young neonatal infants. Research of severe RSV infection in infants is limited by technical difficulty, practical and ethical concerns [9]. Study of RSV infection in animal models requires comparable clinical disease, lesion development, immunity, or other features that relate to the specific aims of the experiment [10]. Animal models for study of human RSV (hRSV) pathogenesis and immune response include rats [11], mice [12], ferrets [13], guinea pigs [14], hamsters [15], lambs [16] and nonhuman primates [17]. Infection in the chimpanzee is viewed by some to be the most analogous to human disease, however, animal availability is limited, few are naive and they require specialized facilities and management making this model impractical [18]. Mice are often used in models for human disease (including RSV) because of the ease of handling and availability of strains with specific knockout genes [12]. However, hRSV infection in rodents produces only mild bronchitis without overt respiratory disease making distinctions about severe infection difficult.

Late term fetal and preterm lambs have proven valuable as pulmonary models for the study of surfactant expression and regulation [19], ventilation-induced injury [20], congenital diaphragmatic hernia [21], chorioamnionitis [22], sleep apnea [16] and persistent pulmonary hypertension of the newborn [23]. A preterm lamb model of hRSV was previously evaluated for RSV-induced sleep apnea and infection
yielded only mild clinical disease [16]. An analogous virus model is bovine RSV (bRSV), a pneumovirus that is genomically, antigenically and functionally related to hRSV and is host-adapted for ruminant infection causing significant pulmonary lesions and clinical disease [18, 24, 25]. The preterm lamb model of bRSV infection has not been assessed for use as a model of RSV disease and lesions. Previous work in the sheep suggested bRSV infection may cause more severe lesions in neonatal versus 6-month old lambs [25, 26]. Furthermore, study of hRSV infection in senile cotton rats suggested age-dependent mechanisms for RSV clearance [11]. Since developmental age is a determinant in severity of RSV disease and lesions, bRSV infection in the preterm lambs could be a novel model for studying the severity of RSV disease in preterm infants. The hypothesis of this study was that preterm lambs are susceptible to bRSV infection and that bRSV activity would be age dependent in the perinatal lamb.

2. Materials and methods

2.1. Animals

Healthy, date-mated commercial ewes were acquired from Iowa State University's Laboratory Animal Services. A gestational age of 138 days (term - 147 days) was chosen to provide a preterm lamb which would not require mechanical ventilation, a known instigator of pulmonary injury [20]. Following removal via surgical uterotomy, lambs were given tactile stimulation with manual chest compressions (if necessary) until respiration was self-regulated, then dried and
placed in a thermo-regulated pen. Within the first hour, fresh colostrum (~200 ml) was given via a stomach tube and ceftiofur (2.2 mg/kg/day, intramuscular) was given to prevent potential bacterial complications [27]. Lambs were hand-fed commercial milk replacer four times daily and milk consumption recorded. In addition, body temperatures and respiration rates were recorded at the same time each day following the morning feeding. Lambs were inoculated at one day of age as follows: the cervical midline was sanitized with 70% ethanol and the trachea isolated for intratracheal injection (20 cc's) of viral inoculum (n=6) or sterile media (n=6).

In addition, eleven neonatal lambs (2-4 days of age) were acquired from Iowa State University's Laboratory Animal Resources and inoculated with bRSV similarly to the preterm lambs one day following arrival. Five of the lambs were inoculated with the same virus inoculum as preterm lambs, three lambs received high-titered viral inoculum and the remaining three received sterile media as a control.

2.2. Virus

Bovine RSV (bRSV) strain-375 was grown in flasks containing adherent bovine turbinate cells (5 % CO₂ and 37 °C). When 90% of virus-induced cytopathic effect was visible (usually within 7 days), the flasks were frozen (-80 °C) and then thawed the next day. All flask media was pooled, thoroughly mixed, sterile filtered and aliquoted. The aliquots were stored at -80 °C. One aliquot was used to determine the tissue culture infective dose (TCID₅₀/ml) by standard plaque assay and the virus titer ranged from 10³-4 TCID₅₀/ml. For the second group of neonatal
lambs, further passages were made to produce a high-dose inoculum with a titer $10^7$ TCID$_{50}$/ml.

2.3. Tissue

On day 7 of infection, lambs (preterm and neonatal) were euthanized with an intravenous injection of sodium pentobarbital. From our preliminary work with bRSV in neonatal lambs, day 7 was chosen because this is approximately when the most severe lesions are seen and when clearance of RSV antigen is underway [28]. The thorax was opened and the lungs examined for gross lesions. The lungs were then removed from the thorax for subsequent tissue collection. The lung was consistently sampled from the left and right: cranial, middle and caudal lobes. Tissues were placed in 10% buffered formalin and processed routinely for hematoxylin and eosin staining or immunohistochemistry.

2.4. Immunohistochemistry

Sections of lung on silanated glass slides were stained with antibody to RSV antigen using a streptavidin-biotin-peroxidase method developed in our laboratory. Briefly, slides of lung tissue were heated to 58 °C for 20 minutes and deparaffinized through a series of ethanol and xylene baths. Antigen retrieval was performed by applying "Pronase E" (0.1 g Protease XIV [Sigma, St. Louis, Mo.] and 0.1 g CaCl$_2$ per 100 mL TBS pH 7.6) on heated slides for 10 minutes followed by TRIS [pH 7.6] (5x's) and PBS (Biogenex, San Ramon, CA) washes (2x's). Normal swine serum (20 % for twenty minutes) was applied as a non specific blocker followed by primary
antibody (mouse monoclonal anti-BRSV-4 [courtesy of Dr. Ken Platt, Iowa State University], 1:100, with normal swine serum [5%] and normal goat serum [5%]) for 72 hours at 4°C. Slides were warmed to room temperature followed by multiple PBS washes. Endogenous peroxidases were blocked by a forty minute application of hydrogen peroxide (3%) in PBS and then repeated PBS washes. Secondary antibody (1:300 biotinylated goat anti-mouse [Kirkegaard & Perry Laboratories, Gaithersburg, MD] with normal swine serum [15%]) was applied for 45 minutes followed by multiple PBS washings. Next the slides were treated with streptavidin-conjugated horseradish peroxidase (Biogenex, San Ramon, CA, 45 minutes), multiple PBS washes, and the chromogen Nova Red (Vector, Burlingame, CA, 5 minutes). The slides were washed in PBS, counterstained with Harris' hematoxylin, dehydrated through a series of ethanol and xylene baths and cover slipped.

2.5. Morphometry

The purpose of the morphometry was to quantify the number of cells staining for bRSV antigen in lesions. Sections of lung from the right and left cranial lobes were used for the study. Since bRSV lesions and immunoreactivity were principally in and around small bronchioles, a pathologist (blinded from the study) randomly selected from low power bronchioles within lesions. At higher magnification, a grid [29] was centered over the bronchiole and adjacent tissue with the total number of positive cells and morphological cell-type being recorded (area = 440 x 440 μm [10^{5.3} μm^2]). At this same time counts for syncytial cells and mitosis were performed. A
total of ten bronchiolar lesions were counted from the left and right sides and averaged.

2.6. Statistics

For clinical data the group means on each day were analyzed for significance using pair-wise t-tests. In the morphometry experiments, the resulting data was analyzed using the Wilcoxon-Mann-Whitney test. For all data, significance was placed at p < 0.05.

3. Results

Clinical scores for the lambs included body temperature, respiration rate and milk consumption (Table 1). The infected preterm lambs had slightly higher temperatures than control lambs throughout the infection with significantly higher temperatures on days two and three of infection (p<0.05). In the infected group, temperatures peaked on day two to three of infection with a decrease until day five with another slight increase starting on days six to seven of infection. From day three through the course of the infection, respiratory rates for the infected group were slightly higher than controls with significant elevation on days five and six of infection (p<0.05). Milk consumption was slightly reduced in the bRSV group through the course of the infection. Infected lambs had a mild cough most noticeable upon exertion.

In regards to preterm lambs and susceptibility to bRSV, all of the infected lambs developed lung lesions while the control lungs were normal. Grossly, the
lungs had multiple plum-red foci (2 - 10 mm) of consolidation that was slightly depressed from the adjacent pleural surface. On cut-surface, the lesions were slightly firm with well-defined borders. The lesions were most evident on the ventral half of the lungs with accentuation in the cranial and hilar regions. Adjacent to the consolidated lesions were pale-pink hyperinflated areas of lung.

Microscopically, there were multifocal areas in which alveolar septa were thickened by macrophages, lymphocytes, plasma cells with small numbers of neutrophils and moderate vascular congestion (Fig. 1-3). Intralesional bronchioles had luminal neutrophils, sloughed epithelial cells and lesser amounts of macrophages with eosinophilic cellular and karyorrhectic debris (Fig. 4). Bronchiolar epithelium was often thickened by hyperplasia of epithelial cells, many of which had mitotic figures. There were also multiple moderate infiltrates of neutrophils and areas of necrosis. In addition, bronchioles had a small number of individual apoptotic cells usually within cytoplasmic vacuoles of adjacent epithelia or mononuclear cells. A small number of the epithelial cells were multinucleated (as syncytial cells) and/or contained small (3-5 μm) intracytoplasmic eosinophilic structures consistent with viral inclusion bodies.

Immunohistochemistry was used to confirm the presence of bRSV antigen and localize the sites of cellular replication. Sections of control lung were consistently negative. In infected lung, all levels of bronchiolar epithelium were immunoreactive for bRSV antigen including a few basal cells to several sloughing epithelial cells (Fig. 5). Within lesions, a few mononuclear cells were immunoreactive and most had a vacuolated to foamy cytoplasms that were morphologically
consistent with macrophages. The granular cytoplasmic staining of immunoreactive epithelial and mononuclear cells was characterized by small globular structures (~0.5 to 8 μm) within the cytoplasm and was consistent with viral inclusions. A smaller number of neutrophils contained antigen. These were often degenerate, found only in the lumen of connecting airways and lack distinct globular structures (inclusions) seen in other infected cells.

Cellular immunoreactivity, syncytial cell formation and mitotic index were used to assess RSV activity and host repair in the preterm versus neonatal lamb. The preterm lambs had more cellular reactivity for bRSV antigen than did neonatal lambs with similar inocula (p<0.05) or high-titer inocula (p<0.05) (Fig. 6, A). Two out of the five lambs in neonatal group 1 (similar virus titer) and one out of three lambs in neonatal group 2 (high virus titer) lacked evidence of immunoreactivity while all of the preterm infected lambs had ample bRSV antigen. The syncytial cell formation, indicative of RSV activity, was significantly higher in the preterm group compared to either of the neonatal bRSV groups (p<0.05) (Fig. 6, B). The mitotic rate, an indicator of reparative hyperplasia, was similar between the different groups (Fig. 6, C).

4. Discussion

The hypothesis of this study was that preterm lambs would develop bRSV disease and that bRSV antigen distribution at the time of clearance would be more widespread in preterm versus full term neonatal lambs. The RSV infected preterm lambs showed clinical evidence of infection including elevated temperature and increased respiration rate with mild loss of appetite (milk consumption) and cough.
The pyrexia and tachypnea are consistent with previous work in older neonatal lambs with hRSV or bRSV infection [16, 25]. Infants with severe hRSV infection typically exhibit a low-grade to moderate fever, croup-like cough and tachypnea with anorexia, lethargy or irritability [3]. The clinical signs in the preterm lambs paralleled many features of severe hRSV infection in infants but notably lacked the severe croup-like cough. This lack of severe cough is similar to previous work in lambs with bRSV [30], but may also be, in part, a consequence of the mid-tracheal inoculation which would have minimized exposure of the upper tracheal and laryngeal epithelium to bRSV for development of laryngotracheitis.

All infected lambs had gross lesions while the controls lacked lesions. The cranioventral to hilar distribution of the lesions was likely a partial result of the aspiration-like allocation of viral media following inoculation and is similar to other work [28]. Gross lung lesions were composed of multifocal atelectasis and consolidation with hyperinflation. Infants with severe hRSV infection can have radiographic evidence of interstitial lung patterns with foci of atelectasis, peribronchiolar thickening and hyperinflation which corresponds to lesions in this study [31].

Histologically, a bronchointerstitial pattern was seen in multiple foci with primarily a mononuclear (macrophages and lymphocytes) infiltrate and congestion in alveolar septa. Severe RSV infection in infants is associated with elevated chemokine levels including RANTES (CC chemokine ligand 5 [CCL5]) and macrophage inflammatory protein-1α (CCL3) which are chemotactic for monocytes.
and other leukocytes [31-33]. RSV-induced chemokine expression likely provided the means for the mononuclear leukocyte infiltrate.

The intralesional bronchioles were characterized by epithelial sloughing, necrosis, and hyperplasia with infiltrates of neutrophils. Leukocyte populations of the connecting airway in severe RSV infection are typically composed of neutrophils and they participate in the viral immunopathology [34]. Epithelial sloughing and necrosis is, in part, a direct result of neutrophil adhesion and exocytosis [35]. RSV infection of epithelial cells and monocytes leads to a synergistic up-regulation in expression of interleukin-8 (IL-8), a CXC chemokine that enhances neutrophilic chemotaxis, and enhanced IL-8 expression is correlated with severe RSV infection [36, 37]. An additional source for epithelial cellular debris is apoptosis, a common mechanism for clearance of RSV infected epithelia [27].

Adjacent bronchiolar epithelium was hyperplastic which is indicative of epithelial repair [38]. The mitotic rate, an indicator of the degree of hyperplasia, was similar between the preterm and neonatal lambs. This present study suggests reduced repair capacity is not likely a factor in severe RSV disease.

Staining for bRSV antigen was present only in infected lambs and was localized to the bronchiolar epithelium with some mononuclear cells. Recently, putative receptor(s) for RSV were described to have L-selectin-like properties [39]. L-selectin is a member of the adhesion-molecule selectin family and is important in leukocyte tethering for endothelial transmigration during normal trafficking or at sites of inflammation [40]. One of the proposed RSV receptors was further characterized as annexin II [39]. Annexin II belongs to the annexin family of calcium- and
phospholipid-binding proteins. In the lung, annexin II is selectively expressed in basal cells, but not columnar cells of the respiratory epithelium [41]. Infection of basal cells would be advantageous for RSV biologically in allowing adequate time during normal cell turnover for viral replication. While a few basal cells contained antigen, most staining was in the sloughing epithelial cells which may correspond to the chronological turnover of previously infected basal cells at inoculation.

The immunoreactivity in mononuclear cells and neutrophils could be due to direct virus infection or phagocytosis of infected cellular debris. Direct infection is possible via putative receptors such as L-selectin-like moieties found on most leukocytes or annexin II which has been localized to monocytes [40, 42]. Ultrastructurally, macrophages of calves infected with bRSV show viral inclusions in the cytoplasm consistent with infection [43]. Most of the mononuclear cell staining in this current study was morphologically consistent with macrophages, and given the intracytoplasmic viral inclusions, direct RSV infection of macrophages was most likely. Other mononuclear cells such as dendritic cells and lymphocytes cannot be excluded as in vitro studies have shown capacity for RSV uptake [44, 45]. As for neutrophil antigen accumulation, phagocytosis of sloughed infected cellular debris in airways probably allowed for much of the neutrophil staining since there was a lack of distinct cytoplasmic inclusions and the quick demise of the degenerate neutrophil following exocytosis into the airway lumen would prevent opportunity for adequate viral replication.

Preterm lambs had significantly more immunoreactive cells and syncytial cell formation than did neonatal lambs with similar (10^{3-4} TCID_{50}/ml) or even higher (10^7
TCID_{50}/ml) virus inoculum. The timing of RSV clearance as assessed by virus isolation, closely parallels staining for bRSV antigen by immunohistochemistry [28]. Syncytial cell formation is, in part, a by-product of the RSV fusion (F) protein and an indicator of RSV activity [46]. The increased bRSV immunoreactivity and syncytial cell formation in the preterm lambs are indicative of a reduced capacity to clear the virus compared to the neonatal lambs. Similar age-dependent differences in viral clearance were seen while studying severe hRSV disease as senile versus young adult cotton rats were less efficient at viral clearance [11]. Reduced RSV clearance in the preterm infant would prolong and perhaps exacerbate inflammation leading to severe clinical disease.

Viral clearance in bRSV infected calves is primarily mediated through apoptosis of infected cells with phagocytic removal of the apoptotic bodies by adjacent epithelial cells or monocytic cells [27]. In cultured respiratory epithelia, RSV infection directly results in expression of many pro- and anti-apoptotic factors. The intracellular balance of these factors may be pushed towards apoptosis through external mononuclear-induced cytotoxicity by binding of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to death receptor-4/-5 (DR4/5) [47]. TRAIL is quickly expressed on monocytes following interferon-α or -γ activation (common with RSV infection) and subsequent binding of DR4/5 expressing cells causes induction of apoptosis [48]. Interestingly, lamb monocytes infected with bovine RSV have decreased phagocytosis and antigen presentation ability [49]. In addition, preterm lamb monocytes have delayed hydrogen peroxide production and reduced phagocytosis of apoptotic cells compared to monocytes from neonatal lambs [50].
Decreased monocytic function in the RSV infected preterm infant may contribute to lack of RSV clearance and thus allow for severe RSV disease.

In this study, we highlight the novel use of bRSV infection in the preterm lamb, which exhibits clinical signs and comparable lesions to severe hRSV disease in the preterm infant. Furthermore, preterm lambs have reduced capacity for RSV antigen clearance compared to neonatal lambs. The preterm lamb model is useful to study age-dependent differences seen in severe RSV infection of preterm infants.

5. Acknowledgments

The authors would like to thank Rachel Derscheid, Erin Costello and Dr. Vanessa Preast for technical assistance. This work was funded by a USDA/CSREES/NRI/CGP grant, award number 2003-35204-13492.

6. References


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Table 1. Average body temperature, respiration rate and daily milk consumption by control and RSV infected preterm lambs.

<table>
<thead>
<tr>
<th>Day of infection</th>
<th>Group</th>
<th>Temperatures (°C +/- sem)</th>
<th>Respiratory rate (respirations/minute +/- sem)</th>
<th>Daily milk consumption (milliliters +/- sem)</th>
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<tr>
<td>Day 1</td>
<td>Control</td>
<td>39.44 +/- 0.17</td>
<td>61 +/- 6.3</td>
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<tr>
<td></td>
<td>RSV</td>
<td>39.69 +/- 0.11</td>
<td>57 +/- 1.3</td>
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<tr>
<td>Day 2</td>
<td>Control</td>
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<td>64 +/- 3.3</td>
<td>563.3 +/- 102.0</td>
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<tr>
<td></td>
<td>RSV</td>
<td>39.98 +/- 0.05*</td>
<td>60 +/- 2.2</td>
<td>498.3 +/- 47.8</td>
</tr>
<tr>
<td>Day 3</td>
<td>Control</td>
<td>39.57 +/- 0.13</td>
<td>60 +/- 3.8</td>
<td>607.5 +/- 84.6</td>
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<td>39.95 +/- 0.05*</td>
<td>65 +/- 2.9</td>
<td>583.5 +/- 78.3</td>
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<tr>
<td>Day 4</td>
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<td>657.5 +/- 80.5</td>
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<tr>
<td>Day 5</td>
<td>Control</td>
<td>39.59 +/- 0.11</td>
<td>50 +/- 3.0</td>
<td>841.7 +/- 100.3</td>
</tr>
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<td></td>
<td>RSV</td>
<td>39.63 +/- 0.16</td>
<td>68 +/- 5.0*</td>
<td>676.7 +/- 63.3</td>
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<tr>
<td>Day 6</td>
<td>Control</td>
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<td>52 +/- 4.0</td>
<td>892.5 +/- 91.1</td>
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<td>RSV</td>
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<td>64 +/- 3.3*</td>
<td>764.2 +/- 69.7</td>
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<tr>
<td>Day 7</td>
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<td>39.87 +/- 0.15</td>
<td>55 +/- 1.8</td>
<td>785.8 +/- 103.6</td>
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</table>

* RSV group compared to control group (p < 0.05)
Fig. 1: Control lungs from lambs given sterile media lacked lesions, bar = 500 μm.

Fig. 2: bRSV-infected lungs had multiple foci of bronchointerstitial consolidation, bar = 500 μm.
Fig. 3: Within lesions, the alveolar septa were thickened by predominantly mononuclear infiltrates, bar = 100 μm.

Fig. 4: Infected bronchiolar epithelium was thickened by hyperplasia with multiple mitotic figures (arrows) and neutrophilic necrotic cellular debris in the lumen. Note the syncytial cell formation (arrowhead), bar = 25 μm.
Fig. 5: Staining for bRSV antigen was primarily within bronchiolar epithelium and a few mononuclear cells (arrow), bar = 25 μm.
Fig. 6: Morphometry of cellular staining for bRSV antigen, syncytial cell formation and mitotic rate in bRSV inoculated preterm (20ml x 10^{3-4} TCID_{50}/ml) and neonatal (20ml x 10^{3-4} TCID_{50}/ml or 10^7 TCID_{50}/ml) lambs. A: The cellular staining for bRSV antigen was greater in the preterm versus either neonate group (p<0.05). All preterm lambs had strong staining while the neonate group had reduced individual staining including two of five lambs in neonate-10^{3-4} TCID_{50}/ml and one of three lambs in neonate-10^7 TCID_{50}/ml which were negative for staining. B: Syncytial cell formation, indicative of bRSV activity, was greater in the preterm lambs compared to either of the neonate groups (p<0.05). C: The mitotic rate, a marker for the degree of reparative hyperplasia, was similar between the preterm and neonatal groups.
CHAPTER 3. DEVELOPMENTAL EXPRESSION AND DISTRIBUTION OF SHEEP BETA-DEFENSIN-2

A paper published in


Abstract

The aim of this study was to define the ontogeny of sheep beta-defensin-2 (SBD-2) mRNA and peptide in selected tissues of fetal, neonatal and adult sheep by real-time PCR and immunohistochemistry, respectively. Fetal and neonatal lambs had a significantly greater SBD-2 tissue distribution than adult sheep. For all ages, the intestines had consistent SBD-2 mRNA expression while extra intestinal expression was sporadic and weak. In adult sheep, SBD-2 mRNA levels decreased from the jejunum caudally to the rectum and a pooled sample from all age groups showed a similar tendency. SBD-2 immunoreactive cells were predominantly in the crypts and base of villi in the small intestine and in a modest number of glands in the large intestine. Interestingly, ileal follicle-associated epithelium lacked detectable SBD-2 immunoreactivity. SBD-2 mRNA and peptide expression are greatest in the intestinal tract and tissue distribution progressively decreases with maturity.
1. Introduction

Antimicrobial peptides are widespread in nature including plant and animal kingdoms and provide innate microcidal protection for the host [1]. Defensins are a subclass of antimicrobial peptides defined by a cationic charge, antimicrobial activity and six to eight highly conserved cysteine residues that form intramolecular disulfide bonds. There are three families of defensins including alpha-, beta- and theta-defensins each characterized by the type of intramolecular disulfide bonds [2].

Beta-defensins have a conserved structure with six-cysteine residues and in humans there are five defined beta-defensin genes. However, a recent computational search has identified an additional 28 putative beta-defensin genes [3]. Beta-defensins have demonstrated antimicrobial activity against several bacteria, fungi and enveloped viruses [1,4,5]. Alterations of the cationic charge or specific amino acid substitutions between defensins correlate with the spectrum of antimicrobial activity. The distribution of beta-defensins at mucosal surfaces (respiratory, gastrointestinal, urogenital, etc) and glands make them an important first line of defense against pathogens [6].

Recently, two sheep beta-defensins (sheep beta-defensin-1 [SBD-1] and sheep beta-defensin-2 [SBD-2]) genes were identified and mapped to sheep chromosome 26 [7]. An additional study of SBD-2 mRNA expression in the gastrointestinal tract of various aged sheep suggested developmental regulation of SBD-2 before and after birth [8]. SBD-2 mRNA tissue distribution, immunohistochemical localization and developmental expression has not been fully
The purpose of this study was to define SBD-2 expression and distribution in select ovine tissues during ontogeny.

2. Materials and Methods

2.1 Animals

Three fetuses (120-140 days gestation [145 days term]), three neonates (3-5 days of age) and three adult sheep with no clinical disease were used for this study. Individual animals were euthanized by intravenous sodium pentobarbital as approved by the animal care and use committee. Tissues collected included nasal turbinate, trachea, lung, rumen, abomasum, jejunum, ileum, spiral colon, rectum, liver, gall bladder, urinary bladder, kidney, uterus (adult) and placenta (fetus). Tissues were both placed in 10% buffered formalin for immunohistochemistry, and snap-frozen in liquid nitrogen for Reverse Transcriptase synthesized cDNA-catalyzed fluorogenic TaqMan real-time PCR (hereafter - real-time PCR).

2.2 Ribonucleic acid isolation

RNA was isolated from each tissue using Trizol reagent [9] by a procedure that was a slight modification of the protocol suggested by the manufacturer's instructions (Invitrogen). Briefly, 0.3 g of tissue and 3 ml of Trizol reagent were homogenized for 30 seconds and the homogenate was allowed to sit for 5 minutes at room temperature. Chloroform (0.6 ml) was added and the mixture was shaken vigorously for 15 seconds then allowed to sit for 3 minutes at room temperature. This
mixture was microfuged for 10 minutes at 4°C, and the resulting top aqueous layer
was retrieved and mixed with isopropanol (1.5 ml) and allowed to sit for 10 minutes
at room temperature. The solution was again microfuged for 10 minutes at 4°C, the
aqueous/isopropanol layer removed and the visible pellet resolubilized in nuclease-
free HPLC-grade water containing 0.1 mM EDTA. Spectrophotometer
measurements were then taken of each RNA isolate (detection at 260nm and
280nm absorbances of 1:40 sample dilutions) to assess the isolates for purity and
quantity.

2.3 Complementary deoxyribonucleic acid production

Deoxyribonuclease (DNase) treatment of RNA samples was performed to
remove potential genomic deoxyribonucleic acid (DNA) contamination with a
commercially available DNase (RQ1 RNase-Free DNase, Promega) following
manufacturer's directions and using the following thermocycler (GeneAmp PCR
System 2400, Perkin Elmer) conditions: 30 minutes at 37°C (to degrade any
contaminating genomic DNA), program was then halted while RQ1 DNase Stop
solution (Promega) was added to each tube, tubes were vortexed and returned to
the thermocycler to resume with a ten minute incubation at 65°C, and a final safety
hold at 4°C. Immediately after this, reverse transcription was performed with a
commercially available kit (TaqMan Reverse Transcriptase Reagents, PE
Biosystems) following manufacturer's directions. Briefly, 2 µg of DNase-treated RNA
from each sample and control were added to a reaction mixture which contained
final concentrations of 1X TaqMan RT buffer, 5.5 mM MgCl₂, 2 mM
deoxyribonucleoside triphosphate (dNTP) mixture (500 μM each dNTP), 2.5 μM random hexamers, 1.25 U/μl murine leukemia virus (MuLV) RT, 0.4-0.8 U/μl ribonuclease (RNase) inhibitor and HPLC-grade water. The following thermocycler conditions were then used: 10 minutes @ 25° C, 30 minutes @ 48° C and 5 minutes @ 95° C. Resulting cDNA was stored in nuclease-free microcentrifuge tubes at -20° C.

2.4 Primer and Probe Design

Sequence-specific oligonucleotide primers and a fluorescent probe for detection and relative quantification of cDNA corresponding to the target gene (SBD-2) were designed with software (ABI Prism Primer Express, Version 1.5, PE Applied Biosystems) according to the software manufacturer's suggestions, and were engineered to be within the coding sequence of the mRNA. Resultant potential primer and probe sequences were compared to all available DNA sequence databases via the search tool BLAST (Basic Local Alignment Search Tool, Version 1.4, National Center for Biotechnology Information) for similarity, and only unique sequences were used for primer and probe design. SBD-2 nucleic acid sequences included the following:

forward primer - 5’AAGCTGCCGTTGGAAGAAAG3’;
reverse primer - 5’CCCGAAACAGGTGCCAATC3’;
and probe: 5’6FAM-TGTGTGCTGACCAAGTGCCCTGGAACCATGAG-TAMRA3’

(6FAM = Fluorescent reporter dye, TAMRA = Fluorescent quencher dye).
Sequence-specific oligonucleotide primers and a fluorescent probe for detection of cDNA corresponding to the endogenous reference gene, 18S Ribosomal RNA, to which SBD-2 cDNA levels would be normalized, were purchased commercially (TaqMan Ribosomal RNA Control Reagents, PE Biosystems).

2.5 Polymerase Chain Reaction

Five separate 96-microwell plates (PE Biosystems) were designed to enable PCR using cDNA from all sheep, with three replicates run for each sheep, as well as three replicates run of a negative control (DEPC-treated water), on each plate. In addition, on each plate were three replicates each of five progressive 1:5 dilutions of cDNA from a control sheep (Sheep #265) that served in the generation of a standard curve, as well as three replicates containing cDNA from a sheep (Fetus #5, ileum) that served as a calibrator to which all other cDNA values would later be compared during analysis. On each plate, target and endogenous reference PCR wells were run simultaneously for the sheep represented on that plate. The assay composition was as follows: the 50 µl PCR mixtures contained 25 µl of the commercially available master mix (Taqman Universal PCR Master Mix - 2X Concentration, PE Biosystems); 5 µl of a 1:5 dilution of cDNA (or DEPC-treated water for the negative control); SBD-2 forward primer, reverse primer and probe concentrations of 300 nM, 900 nM and 150 nM, respectively, or 50 nM, 50 nM and 200 nM, respectively, for the endogenous control of 18S rRNA (PE Biosystems); and DEPC-treated water. This plate was run in the sequence detection system (GeneAmp 5700 Sequence Detection System, Version 1.3, Applied Biosystems) with conditions identical to
those used in the optimization and validation tests. Resultant data gathered by the
detection system was exported from the machine to floppy disk, and all final data
processing was performed using a departmentally-designed EXCEL file to compare
target to reference cDNA signals in order to create relative mRNA expression
graphs.

2.6 Immunohistochemistry

The SBD-2 primary antibody was generated in rabbits inoculated with a
synthesized SBD-2 peptide. This peptide was purified by high performance liquid
chromatography, assessed by matrix-assisted laser desorption/ionization-mass
spectrophotometry for molecular weight, and in western blots the antibody bound the
synthesized 42 amino acid SBD-2 peptide as well as a band from ileum
homogenates at the appropriate molecular weight of 4.6 kDa. Paraffin-embedded
tissues were cut (6-microns) and placed on glass slides. These were deparaffinized
in xylene followed by a series of ethanol baths and washed (PBS/0.1% Tween 20).
Normal swine serum (30%) was applied for 30 minutes for nonspecific protein block.
Next the primary antibody (polyclonal rabbit anti-SBD-2 [1:5000 in BioGenex Diluent]
with 5% normal goat serum and 5% normal swine serum) was applied to the tissue
in 5-slide plastic mailers for 72 hours at 4° C. Slides were allowed to warm to room
temperature, washed, then a secondary antibody (biotinylated Goat anti-Rabbit
Kirkegaard & Perry Labs), 1:400 in BioGenex Diluent with 25% normal swine
serum) was applied for 45 minutes followed by multiple washing baths. The
chromogen (Vector Nova Red) was applied for five minutes followed by multiple
water washes. The tissues were counterstained with hematoxylin, dehydrated through a series of ethanol and xylene baths and cover slipped with Permount.

2.7 Statistical analysis

Pair wise t-tests were used to assess differences in age/tissue group means. In order to assess the intestinal SBD-2 expression trend, linear regression was used to calculate the sign (+/-) of the slope coefficient. The statistical analysis for SBD-2 mRNA tissue distribution was done with the nonparametric Wilcoxon test.

3. Results

The aim of this study was to define the expression and cellular distribution of SBD-2 in select tissues during ontogeny. We utilized real-time PCR for SBD-2 mRNA tissue expression and immunohistochemistry for SBD-2 cellular distribution.

3.1 Real-time PCR

SBD-2 mRNA levels were highly variable between individual animals even of the same age group. This contributed to the lack of statistical significance in SBD-2 expression among age or tissue group means. The jejunum had the highest SBD-2 mRNA expression for all individuals. In addition all individuals had SBD-2 mRNA consistently expressed in the jejunum, ileum, spiral colon and rectum. In the adult sheep, relative SBD-2 mRNA expression progressively decreased from the jejunum to the rectum (Fig. 1A, p = 0.008). The neonatal lambs had similar progressively decreasing expression in the intestinal tract, however the decrease was not
statistically significant due to the extensive individual variability (Fig. 1B). The fetal group had slightly lower relative expression values than the neonate and adult, but still had marked individual variability (Fig. 1C). When the tissues mRNA levels from all age groups were pooled, a general trend of decreased SBD-2 mRNA expression from jejunum to rectum ($p = 0.052$) was detected.

While the intestinal SBD-2 mRNA was present in all individuals, other tissues had sporadic SBD-2 mRNA expression (Table 1). Extra-intestinal expression was present in the respiratory tract (turbinate, trachea, lung), urinary tract (kidney, urinary bladder), liver and uterus. The SBD-2 mRNA expression in these tissues was often a small fraction of the calibrator and the tissue distribution was variable among individuals. The fetal and neonatal lambs had wider tissue distribution of SBD-2 mRNA than did adult sheep ($p = 0.042$, fetus/neonate lambs vs. adults). No SBD-2 expression was evident by any age group in the rumen, abomasum, gall bladder or fetal placenta.

3.2 Immunohistochemical detection of SBD-2

Tissues with SBD-2 mRNA expression were examined for cellular distribution of SBD-2 peptide immunoreactivity. In jejunum, SBD-2 immunoreactivity was present within the epithelial cells of villus crypts (Fig. 2). Approximately 50% of the crypt cells were immunoreactive and staining was present throughout the cytoplasm in jejunum and other tissues; nuclei lacked immunoreactivity in all cells in all tissues. The number of epithelial cells in jejunum with immunoreactivity progressively decreased from the crypts to the lower base of villi. Enterocytes lining the luminal
two-thirds of the villus epithelium generally lacked immunoreactivity; only an occasional cell had immunoreactivity. Goblet cells located at the base of villi had mild/moderate immunoreactivity while those on the upper villus lacked staining. Ileal immunoreactivity was similar to that of the jejunum except that there were fewer cells with immunoreactivity in the crypts and villus epithelium. In the ileal Peyer's patches, the follicle-associated epithelium (FAE) lacked immunoreactivity (Fig. 3). The spiral colon and rectum both had a few to roughly 30% of glands with immunoreactivity of individual cells. Immunoreactive cells were rarely present in the luminal half of the gland. For all tissues, staining intensity and distribution was highest for the jejunum and progressively decreased to the rectum. This was true for all age groups. Immunoreactivity to SBD-2 peptide was not detected in extra-intestinal tissues with low mRNA expression (data not shown).

4. Discussion

In this study we defined SBD-2 mRNA and peptide expression and distribution in the sheep fetus, neonate and adult. The amount of SBD-2 mRNA expression was highly variable between individuals, even in tissues with consistent expression, such as the intestinal tract. The individual variability of SBD-2 expression was partially characterized in a previous SBD-2 study [8] and the variability of SBD-2 expression is similar to expression of alpha- and beta-defensins in human intestine [10]. Inflammatory cytokines, interactions with microbial pathogens, and cellular proliferation/differentiation are a few of the factors that can regulate beta-defensin expression [1,11,12]. The individual variability of SBD-2 and
other intestinal defensins may reflect a dynamic multifactorial influence by the local environment in defensin regulation and expression.

All individuals had SBD-2 mRNA expression consistently in the intestines and individual expression was highest in jejunum. The immunoreactivity of SBD-2 peptide was highest in the jejunum and corresponded to the jejunal mRNA expression of SBD-2. The relative expression of SBD-2 mRNA in the small and large intestine is similar to that of human alpha-defensins (HD5 and HD6), which are a component of Paneth cell secretory granules [10]. Paneth cells are specialized cells in the crypts of Lieberkühn that can influence the local microbial environment and contribute to the mucosal barrier by their secretions including antimicrobial peptides [13]. In our study, SBD-2 immunoreactivity was principally present in crypt cells that were similar microscopically in location and morphology with Paneth cells. While Paneth cells are a logical source for SBD-2, the presence of stained cells (including goblet cells) at the base of jejunal and ileal villi along with stained cells in the colon and rectum suggests other epithelial cells may be involved. In our study, goblet cells low on the villus were immunoreactive while more mature goblet cells higher on the villus lacked staining. This is consistent with an electron microscopy study showing α-defensin positive granules in immature murine goblet cells but not in mature goblet cells of the upper half of the villus [14]. Immature goblet cells may represent a cellular source for moderate SBD-2 expression that is widely situated throughout the intestines. We also show that SBD-2 mRNA levels decrease caudally from the jejunum to the rectum in adult sheep (p=0.008) and for combined age group (0.052). Similarly, the immunoreactivity was highest in the jejunum with progressive decrease
caudally to the rectum. The decreasing expression from jejunum to rectum could partially be explained by the relative tissue distribution of Paneth cells.

The consistent expression of SBD-2 mRNA in the intestine may reflect an emerging role for beta-defensins in adaptive immunity. Human beta-defensins-1 &-2 are ligands for CC-chemokine receptor-6 (CCR6), a surface receptor of CD45RO+ memory T cells and bone-marrow derived immature dendritic cells [14,15,16]. Altered expression of CCR6 ligand can regulate the trafficking of these cells to the intestines [17, 18]. While interactions between SBD-2 peptide and surface receptors have not been performed, SBD-2 may have similar activity and serve as a human model for investigations of beta-defensin innate and adaptive immune regulation.

An additional observation was the absence of immunoreactivity by the ileal FAE. Peyer's patches and FAE of the sheep ileum are most pronounced in the young animal and by adulthood are significantly regressed [19]. The FAE in the ruminant ileum is composed of a nearly uniform, specialized cell population that resides over lymphoid tissue (e.g. Peyer's patch) on discrete dome villi [20]. These specialized epithelial cells have surface folds that can internalize particulate matter for lysosomal degradation with subsequent exocytosis of the residual bodies [21]. In some species, the FAE has reduced protective mechanisms (glycocalyx and mucus layer) as compared to absorptive epithelium that can facilitate interaction with microbes for antigen sampling [22, 23, 24]. The lack of antimicrobial peptides such as SBD-2 in cells of the FAE may facilitate intimate access by microbes for antigen sampling.
Extra intestinal SBD-2 mRNA was inconsistently and weakly expressed by a wide range of tissues. While SBD-2 mRNA was detected in extra-intestinal tissues by real time PCR, the peptide production was insufficient for detection by immunohistochemistry. The young lambs (combined fetus/neonate) had a significantly wider tissue distribution of SBD-2 mRNA than the adult group. To our knowledge we are the first to report a wider defensin tissue distribution in developing individuals compared to mature ones. While beta-defensins are increasingly known for their role in mucosal immunity, beta-defensins have also been shown to participate in cellular differentiation/growth in vitro [11,25]. A role in cellular growth/differentiation may explain the wider SBD-2 tissue distribution in young developing animals.

This study shows SBD-2 expression and distribution in the fetus, neonate and adult sheep. This provides a foundation for future efforts to examine the inducibility and role of SBD-2 in specific tissues and diseases.

Acknowledgments

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References


FIG. 1. SBD-2 mRNA levels in intestine relative to calibrator tissue. (A) Intestinal expression in adult sheep. (B) Intestinal expression in neonatal lambs. (C) Intestinal expression in preterm lambs (fetus).
FIG. 2. Immunohistochemical detection of SBD-2 peptide in the jejunal crypts of adult sheep. Approximately half of the cells are immunoreactive for SBD-2 peptide. Bar = 50 microns.
FIG. 3. Immunohistochemical detection of SBD-2 peptide in ileum of neonatal lamb, the absorptive epithelium (arrows) has multiple immunoreactive cells (including goblet cells) while the follicle-associated epithelium (arrowheads) lacks immunoreactivity, Bar = 50 microns.
Table 1. SBD-2 mRNA\(^a\) distribution in selected tissues of fetus, neonatal and adult sheep (n = 3/group).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fetus</th>
<th>Neonate</th>
<th>Adult(^b)</th>
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<tbody>
<tr>
<td>Jejunum</td>
<td>3(^c)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ileum</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Spiral colon</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Rectum</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Trachea</td>
<td>2</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Kidney</td>
<td>2</td>
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<tr>
<td>Turbinate</td>
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<td>0</td>
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</tr>
<tr>
<td>Urinary bladder</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Liver</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Uterus</td>
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</table>

\(^a\) Tissues were considered positive by real-time PCR if SBD-2 mRNA was amplified within 45 cycles.

\(^b\) SBD-2 mRNA tissue distribution in the adult group was significantly less than those in the fetus/neonate groups combined (p=0.04).

\(^c\) Represents the number of tissues (0 to 3) with amplified SBD-2 mRNA from each group.
CHAPTER 4. ADENOVIRUS-MEDIATED GENE THERAPY ENHANCES PARAINFLUENZA VIRUS-3 INFECTION IN NEONATAL LAMBS

A paper submitted to the Journal of Clinical Microbiology


Abstract

Parainfluenza viruses are a common cause of seasonal respiratory disease, but in high-risk individuals (e.g. young children) these viruses can cause severe clinical manifestations that require hospitalization. Beta-defensins are a subclass of antimicrobial peptides with antiviral activity. Use of adenoviral-mediated beta-defensin gene expression has been proposed as therapy for chronic bacterial infection commonly seen in cystic fibrosis patients; however, its use during parainfluenza virus-3 (PIV3) infection has not been evaluated. The hypothesis of this experiment was that adenoviral expression of human beta-defensin-6 (HBD6) would diminish concurrent PIV3 infection in neonatal lambs. The PIV3/Ad/HBD6 group had increased recruitment of pulmonary neutrophils compared to the PIV3 or PIV3/Ad groups with increased respiration rate and body temperature late in the course of the PIV3/Ad/HBD6 infection. Interestingly, the adenovirus treated groups had higher PIV3 immunohistochemical staining and syncytial cell formation than did the PIV3 group suggesting that treatment with the adenovirus vector, regardless if carrying a
target gene, exacerbated the PIV3 infection. The mRNA of antimicrobial surfactant proteins A&D and sheep beta-defensin-1 were increased by PIV3 and adenoviral treatment, roughly corresponding to the degree of inflammation. While high dose pulmonary administration of an adenovirus vector has been associated with undesirable inflammation, this is the first study to show that it can exacerbate concurrent viral infection, a concern that needs to be addressed for future adenoviral studies in the lung. Additionally this study showed that adenoviral-mediated HBD6 expression increases neutrophil recruitment, a recently described attribute of beta-defensins, with mild accentuation of PIV3 activity and inflammation.

Introduction

Parainfluenza viruses-1 and -3 are enveloped, single-strand, negative-sense RNA viruses of the Paramyxoviridae family and are an important, ubiquitous cause of seasonal respiratory disease. Infection in a healthy individual is localized primarily to the upper respiratory tract and is often self-limiting (13, 27). However, human parainfluenza viruses can cause more serious respiratory disease such as severe laryngotracheitis ("croupy" cough), bronchiolitis and pneumonia leading to hospitalization and on rare occasions, death (15). Risk factors for severe manifestations of parainfluenza virus infection include: primarily lung disease (e.g. cystic fibrosis or asthma), the immunocompromised (e.g. transplant recipients), young children, and the elderly (9, 15, 16). Parainfluenza viruses, along with other viruses are increasingly recognized in exacerbation of cystic fibrosis lung disease and may predispose to secondary bacterial infection, including *Pseudomonas*
*P. aeruginosa* which is a persistent problem in cystic fibrosis lungs (37). Young children and infants with cystic fibrosis are at increased risk for lower respiratory tract infections by these ubiquitous viruses resulting in increased hospitalization rates and chronic lung damage (16, 36).

Defensins are a conserved class of antimicrobial peptides characterized by a cationic charge, antimicrobial activity and six to eight highly conserved cysteine residues that form intramolecular disulfide bonds. There are three families of defensins including alpha-, beta- and theta-defensins each characterized by the organization of intramolecular disulfide bonds (39). Beta-defensins are a class of cationic antimicrobial peptides that are produced primarily in epithelium at mucosal sites including the lungs (30). There are over thirty putative human beta-defensin genes and many of those gene products already characterized have a wide spectrum of microbicidal activity against bacteria, fungi and enveloped viruses (1, 31). Antiviral action by beta-defensins is thought to be through disruption of the viral envelope, however recent work in alpha-defensins suggests the antiviral activity may also involve intracellular cell signaling to inhibit viral replication (6, 30). Inactivation of beta-defensins by the altered local environment (e.g. altered salinity or airway surface liquid) has been suggested to contribute to the increased susceptibility of cystic fibrosis patients to pulmonary infection (3, 11).

Perinatal lambs are commonly used models for pulmonary disease research including those involving gene therapies (19, 26). Perinatal lambs are susceptible to ovine isolates of PIV3, which have similar genomic and antigenic properties to human PIV3 and causes spontaneous respiratory disease and lesions in sheep (20,
Previous work in the human bronchial xenograft model using adenoviral mediated gene therapy with over-expression of a cathelicidin antimicrobial peptide (LL-37/hCAP-18) showed success in killing bacteria suggesting a use for antimicrobial gene therapy in predisposed patients (4); however, evaluation of antimicrobial peptide gene therapy has not been applied to diminish viral infection. Human beta-defensin-6 (HBD6), a recently described beta-defensin selectively expressed in the epididymis, was chosen as the antimicrobial peptide in this model due to its lack of expression in the human lung or in sheep (38). The hypothesis of this study was that HBD6 gene expression in the neonatal lamb lung would diminish concurrent PIV3 infection in neonatal lambs.

**Materials and methods**

**Animals**

All animal studies were approved by the Iowa State University Animal Care and Use Committee. Neonatal lambs (n=20) were obtained from Iowa State University's Laboratory Animal Resources. At 3-5 days after birth (allowing time for acclimation) the lambs were inoculated intratracheally with two, twenty milliliter volumes at approximately thirty minute intervals. The order and composition of the inoculum for each group (n=4) were as follows: Group A) sterile media/sterile media, (Control group); B) sterile media/PIV-3, (PIV3 group); C) adenovirus vector (no gene insertion)/PIV-3, (PIV3/Ad group); or D) adenovirus-HBD6/PIV-3, (PIV3/Ad/HBD6 group). At day seven of infection the lambs were euthanized by an intravenous injection of sodium pentobarbital for collection of tissues. This time point was chosen
from our preliminary data as the time of significant lesion development and active viral clearance. An additional group of lambs were given an intratracheal inoculation (20 ml) of sterile media (n=2) or an adenovirus/HBD6 (n=2) also containing a lacZ gene insert for cellular localization of transfection. These lambs were euthanized at day 4 of infection which is near the peak for pulmonary adenoviral vector gene expression (10).

Viruses

The ovine isolate of parainfluenza-3 virus was grown as previously described to $10^{7.8}$ TCID$_{50}$/ml (20 ml per lamb) (20). The replication defective human adenovirus-5 vector (Ad) had a cytomegalovirus promoter and was commercially acquired with the human beta-densin-6 (HBD6) gene (IMAGE consortium ID # 2602699), HBD6 with a lacZ reporter gene, or no gene insert (ViraQuest Inc., North Liberty, Iowa). The Ad (no gene insert) and Ad/HBD6 had an E1 deletion (nucleotides 358 to 3328) and the Ad/HBD6/LacZ insert for X-gal staining had deletions at nucleotides 358 to 3328 and 28592 to 30470 with concurrent loss of E1/E3 functions. Each lamb received approximately $10^{12}$ virus particles in 20 ml of sterile saline.

Clinical parameters

The lambs were assessed daily for different clinical parameters. Body weight, temperature, and respiratory rate were taken at the same time of day following the morning feeding. The lambs were treated daily with antibiotic (ceftiofur 10 mg/day, SQ) to prevent complications associated with secondary bacterial infection (34).
Tissues

Lung tissue was collected consistently from the cranial and caudal lobes on the left and right sides. Tissues were placed in 10% neutral-buffered formalin and processed routinely for hematoxylin and eosin staining or immunohistochemistry. In addition, lung tissue was snap frozen in liquid nitrogen for RNA isolation and real time fluorogenic PCR.

Ribonucleic acid isolation

Ribonucleic acid isolation and complementary deoxyribonucleic acid production were performed as previously described (24). Briefly, 0.3 g of tissue and 3 ml of Trizol reagent (Invitrogen) were homogenized for 30 seconds and the homogenate was allowed to sit for five minutes at room temperature. Chloroform (0.6 ml) was added and the mixture was shaken vigorously for fifteen seconds then allowed to sit for three minutes at room temperature. This mixture was microfuged for ten minutes at $4^\circ$C, and the resulting top aqueous layer was retrieved and mixed with isopropanol (1.5 ml) and allowed to sit for ten minutes at room temperature. The solution was again microfuged for ten minutes at $4^\circ$C, the aqueous/isopropanol layer removed and the visible pellet resolubilized in nuclease-free HPLC-grade water containing 0.1 mM EDTA. Spectrophotometer measurements were then taken of each RNA isolate (detection at 260nm and 280nm absorbances of 1:40 sample dilutions) to assess the isolates for purity and quantity.
Complementary deoxyribonucleic acid production

RNA samples were treated with a commercially available deoxyribonuclease (DNase) (RQ1 RNase-Free DNase, Promega) to remove potential genomic deoxyribonucleic acid (DNA) contamination. Immediately after this, reverse transcription was performed with a commercially available kit (TaqMan Reverse Transcriptase Reagents, PE Biosystems) following manufacturer’s directions. Briefly, 2 μg of DNase-treated RNA from each sample and control were added to a reaction mixture which contained final concentrations of 1X TaqMan RT buffer, 5.5 mM MgCl₂, 2 mM deoxyribonucleoside triphosphate (dNTP) mixture (500 μM each dNTP), 2.5 μM random hexamers, 1.25 U/μl murine leukemia virus (MuLV) RT, 0.4-0.8 U/μl ribonuclease (RNase) inhibitor and HPLC-grade water. The following thermocycler conditions were then used: 10 minutes @ 25° C, 30 minutes @ 48° C and 5 minutes @ 95° C. Resulting cDNA was stored in nuclease-free microcentrifuge tubes at 20° C.

Primer and Probe Design

Sequence-specific oligonucleotide primers and a fluorescent probe were designed with software (ABI Prism Primer Express, Version 1.5, PE Applied Biosystems) according to the software manufacturer’s suggestions. Resultant potential primer and probe sequences were compared to all available DNA sequence databases via the search tool BLAST (Basic Local Alignment Search Tool, Version 1.4, National Center for Biotechnology Information) for similarity, and only unique sequences were used for primer and probe design (Table 1). Sequence-
specific oligonucleotide primers and a fluorescent probe for detection of cDNA corresponding to the endogenous reference gene, 18S Ribosomal RNA, to which target cDNA levels would be normalized, were purchased commercially (TaqMan Ribosomal RNA Control Reagents, PE Biosystems).

Polymerase Chain Reaction

The 96-microwell plates were designed to enable two replicates for both the target gene and endogenous 18S ribosomal RNA of: 1:5 dilution cDNA from all lambs, negative control (DEPC-treated water), and five progressive 1:5 dilutions of cDNA from a control sheep for generation of the standard curve. On each plate, target and endogenous reference PCR wells were run simultaneously for the tissues represented on that plate. The plate was run in the sequence detection system (GeneAmp 5700 Sequence Detection System, Version 1.3, Applied Biosystems) with conditions identical to those used in the optimization and validation tests. Resultant data gathered by the detection system was exported from the machine to floppy disk, and all final data processing was performed using a departmentally-designed EXCEL file to compare target to reference cDNA signals in order to create relative mRNA expression graphs.

Parainfluenza virus-3 immunohistochemistry

Formalin-fixed, paraffin embedded sections were deparaffinized in xylene followed by a series of alcohol baths and Pronase E (0.1 g Protease XIV and 0.1 g CaCl₂ per 100 mL TBS pH 7.4) was applied for antigen retrieval. After washing
(PBS), the background blocker (20% normal swine serum) followed by primary antibody (goat polyclonal anti-bovine parainfluenza virus-3, 1:1000, [VMRD]) was applied to the tissues. These were washed, hydrogen peroxide (3% in methanol) applied to block endogenous peroxidases and washed again. Secondary antibody (1:400 biotinylated rabbit anti-goat) was applied followed by washing, SS-HRP and chromogen (Vector Red, Vector). The slides were counterstained with hematoxylin, dehydrates in a series of alcohol baths then xylene and cover slipped with Permount.

Morphometry

Measurement of microscopic morphological changes including cellular staining, mitotic rate, neutrophil density, and syncytial cell formation were assessed by a pathologist blinded from the study using a grid (19). Cranial and caudal lung lobes (bilaterally) were used for the study. For each slide, the pathologist randomly chose ten sites from low power, then the selected morphological alteration was counted at high power. This was done for each segment of lung, the results were totaled and averaged to the grid area (13,000 or 52,000 μm²).

Adenovirus distribution

OCT embedded frozen sections were cut at 6 μm and placed in a bath of 4% formaldehyde for 5 minutes and the slides were then washed twice in HBSS. X-gal staining solution (20 mg [5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside] per ml of N’N’-Dimethylformamide) was added (20µl/ml) to a fresh solution of 5mM
potassium ferricyanide, 5mM potassium ferrocyanide, 2 mM magnesium chloride in HBSS for the final X-gal working solution. Slides were then exposed to the final X-gal working solution for 10 hours at 37°C. The slides were washed in HBSS counter stained with nuclear fast red and cover slipped. Sections were then examined for cells staining blue indicative of LacZ expression (24).

Statistics

For assessment of clinical and fluorogenic real-time PCR data, significance was determined to be $p<0.05$. Non-parametric Wilcoxon tests were used to assess for statistical differences in unequal sized samples. T-tests assuming unequal variances were used to assess for differences in the means of groups of equal size.

Results

The daily body temperatures showed early and late increases through the course of infection. On day two, body temperatures for all PIV3 infected groups were generally increased with the PIV3/Ad/HBD6 having a significantly higher temperature than the control ($p<0.01$, Figure 1). Starting on day five the PIV3/Ad/HBD6 group had significantly increased body temperature over controls that continued through the duration of the experiment ($p<0.05$). The PIV3 and PIV3/Ad had moderately increased temperatures that were not statistically significant except for the PIV3 group on day 6 ($p<0.01$).

The respiration rate on day two was significantly higher in the PIV3/Ad group ($p<0.05$) and moderately elevated in the PIV3/Ad/HBD6 group ($p=0.08$) compared to
the PIV3 group (Figure 2). By day four through the course of infection the PIV3 infected groups were generally elevated (p<0.05) above the controls, except for an increase that lacked statistical significance for the PIV3/Ad/HBD6 group on day 6. Interestingly, the PIV3/Ad/HBD6 group had a slightly higher respiration rate on day six and seven than did the PIV3 or PIV3/Ad group.

Daily milk consumption (liters per kilogram body weight) was evaluated to determine if there were any clinical differences in appetite. For the duration of the experiment the PIV3, PIV3/Ad, and PIV3/Ad/HBD6 groups had decreased (p<0.05) average milk consumption compared to the control group with no significant difference among the PIV3 treated groups (Figure 3).

Growth of the lambs (average daily gain, kg/day) during the experiment corresponded to the milk consumption with significantly decreased growth in the PIV3 (p<0.05) and PIV3/Ad/HBD6 (p<0.05) groups (Figure 4). The PIV3/Ad showed a similar decrease in growth but was not statistically significant.

Grossly, the control lambs had no visible lesions while the PIV3, PIV3/Ad and PIV3/Ad/HBD6 groups had a cranial-ventral to hilar distribution of multifocal plumbeous consolidation. There was a slightly wider lesion distribution in the PIV3/Ad and PIV3/Ad/HBD6 groups over the PIV3 group. Microscopically the PIV3 lesions consisted of multifocal thickening of the alveolar septa by macrophages, and a small number of lymphocytes, neutrophils and plasma cells. The bronchiolar lumens contained small aggregates of neutrophils and sloughed cellular debris. Within these areas, the bronchiolar epithelium was thickened due to hyperplasia with folds of piled cells having pale cytoplasm, increased mitotic figures and infrequent syncytial
cells. The PIV3/Ad/HBD6 group appeared to have relatively higher numbers of neutrophils within lung when compared to lambs inoculated with PIV3 alone.

To evaluate neutrophil recruitment to the lungs following treatment, pulmonary neutrophil density was determined by morphometry. There was a significantly increased neutrophilic infiltrate in each of the PIV3 infected groups over the sterile media controls (p<0.05, Figure 5). In addition, the PIV3/Ad/HBD6 group had a higher neutrophil count than did either of the PIV3 or PIV3/Ad groups (p<0.05).

PIV3 immunohistochemistry produced no staining in the control lungs while cellular staining was present in all lambs of the PIV3 infected groups. This was characterized by small granular red staining within the cytoplasm (Figure 6). Affected cells most often were epithelial cells of the distal bronchi to terminal bronchioles. Morphometrical quantification of the cellular PIV3 immunostaining showed increased immunoreactive cells in PIV3/Ad (p<0.05) and PIV3/Ad/HBD6 (p<0.05) groups compared to the PIV3 group (Figure 7).

The mitotic rate was significantly increased in all of the PIV3 infected groups over the control group (Figure 8). No significant changes within the PIV3 infected groups were evident. PIV3-induced syncytial cell formation was not present in the control group but was present in all of the PIV3 infected groups. The combined adenoviral gene therapy groups had higher syncytial cell density than did the PIV3 infected lambs (p<0.05, Figure 9).

The adenovirus vector was localized by X-gal staining to a small number (<5%) of individual epithelial cells of the bronchi and terminal bronchioles (data not
shown). This staining was not seen in samples from two control lambs inoculated with sterile media.

Real-time fluorogenic polymerase chain reaction was used to assess for transfection of HBD6 and for alterations in host mRNA of SBD1, SP-A and SP-D. For HBD6, the control (PIV3/Ad inoculated) groups had no fluorogenic amplification, while all four of the HBD6 inoculated lambs had late-cycle amplification indicating weak HBD6 expression (data not shown). Expression of SP-D in each PIV3 infected group was significantly elevated over the control group and the expression levels tended to increase from the PIV3/Ad group to the PIV3/Ad/HBD6 group (Figure 10). Similar trends were present in the SP-A and SBD1 mRNA expression with statistical significant seen in the combined PIV3/Ad and PIV3/Ad/HBD6 groups versus the PIV3 group for both SP-A and SBD1 (p<0.05, Figures 11 and 12).

Discussion

The hypothesis of this study was that beta-defensin (HBD6) expression during concurrent PIV3 infection would diminish the disease progression. Clinical parameters were monitored during the course of infection and day seven of infection was chosen for necropsy as lesions are usually marked with active viral clearance in lambs (7, 20). All PIV3 infected groups had clinical evidence of infection (e.g. elevated temperature and tachypnea, depresses milk consumption and growth); gross and microscopic lesions consistent with PIV3; and immunohistochemical staining in PIV3-infected animals only. Adenovirus transfection was confirmed with
X-gal staining in Ad/HBD6/LacZ infected airway epithelium on day four and by HBD6 mRNA expression on day seven of infection.

The adenovirus treated groups (PIV3/Ad and PIV3/Ad/HBD6) had increased temperatures and respiration rates on day two of infection. This was consistent with previous work in which BALB/c mice showed increased proinflammatory cytokines of tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) within even hours of human adenovirus 5 intrapulmonary inoculation, (40). TNF-alpha and IL-6 are endogenous pyrogens accounting for the increased temperature and TNF-alpha mediates bronchial and vascular smooth muscle contractility which along with pyrexia could have increased the respiratory rate (2, 35). Other clinical alterations compared to the controls were generally attributable to PIV3 infection. Of particular interest was that the adenoviral treated groups had increased PIV3 staining and syncytial cell formation than did the PIV3 groups lacking adenoviral treatment. Antigen staining occurs at sites of PIV3 protein synthesis and syncytial cell formation is mediated by the fusion (F) protein with regulation by the hemagglutinin-neuraminidase (HN) protein, both indicators are byproducts of PIV3 replication (28).

The increased immunostaining and syncytial cell formation were both indicative of enhanced PIV3 viral activity (21, 28). This novel finding suggests that pulmonary adenoviral mediated gene therapy can exacerbate concurrent PIV3 infection. Proteins of adenoviruses, including E1A, E1B and E3 proteins, have been shown to have immunomodulating functions most of which are immunosuppressing (17, 29). The vector used in this study had an E1 deletion with the E3 gene intact. E3 gene products inhibit: peptide processing for MHC I presentation; activation of
nuclear factor-kappaB (NF-κB); and apoptosis through FAS, TRAIL and TNF-alpha pathways (5, 8, 17, 29, 32). MHC I processing and apoptosis of virus infected cells are important mechanisms for viral clearance and the synthesis of antiviral type I interferon along with other inflammatory mediators are mediated in part through NF-κB activation. Early E3 inhibition of antiviral pathways could have allowed for more enhanced PIV3 replication and infection. While immunosuppressive effects of adenovirus E3 gene may participate in enhancing PIV3 infection, the small cellular distribution of the adenovirus suggests this may not be a significant component. Another mechanism for the enhanced PIV3 infection may be the general inflammatory and cellular changes caused by the addition of the adenovirus vector. In previous work, pretreatment of 4-ipomeanol similarly enhanced PIV3 activity in a calf model (21). The 4-ipomeanol compound is biotransformed in Clara cells into a free radical metabolite which causes necrotizing bronchiolitis and interstitial pneumonia. The authors of the PIV3 calf model hypothesized that 4-impomeanol may 1) suppress pulmonary innate defenses; 2) increase the number of susceptible cells; or 3) increase factors (e.g. proteases) for enhanced viral replication. Early immunosuppression by adenoviral proteins and/or altered cellular susceptibility to PIV3 infection/replication may contribute to this increased PIV3 activity in adenovirus treated lambs. Further work needs to clarify and define the mechanism.

We further evaluated the mRNA levels of innate antimicrobial peptides and proteins to determine if adenoviral gene therapy altered mRNA expression. SP-D was increased for each PIV3 group over the control group (p<0.05) with moderate increases in the PIV3/Ad/HBD6 group over the PIV3/Ad and PIV3 groups. Similar
trends were seen for SP-A and SBD1 with significant increases in the combined PIV3/Ad and PIV3/Ad/HBD6 group over the PIV3 groups (p<0.05). Surfactant protein and beta-defensin mRNA expression is partially regulated by the status of pulmonary inflammation and lung injury (22, 30). The increases seen here are likely a coordinated response to exacerbated inflammation and injury associated with inoculation of adenoviral vector and exacerbated PIV3 viral infection.

The effect of in vivo HBD6 expression on PIV3 infection was unanticipated as it tended to be proinflammatory, aggravating to the overall clinical situation. Microscopically, neutrophil infiltration was increased in the PIV3/Ad/HBD6 group and this was confirmed with morphometry in which the neutrophils density was increased (p<0.05) over either PIV3 or PIV3/Ad groups. Clinical scores of body temperature and respiration rates for PIV3/Ad/HBD6 groups were increased over control with slight increases over the PIV3/Ad group. Beta-defensins, while increasingly known for their antimicrobial properties, have additional functions such as leukocyte chemotaxis and immunomodulation (30). Recently, HBD2 was shown to function in vitro as a chemoattractant for TNF-alpha primed neutrophils and HBD2 ligand interaction with CC-chemokine-receptor-6 (CCR6) was considered the mechanism for chemotaxis (25). TNF-alpha expression is an early consequence of pulmonary adenovirus mediated gene therapy and may prime the neutrophils for CCR6 mediated chemotaxis (40). The HBD6 expression in these lambs stimulated both increased neutrophilic infiltrate and accentuated clinical parameters of PIV3 disease without decreasing PIV3 activity, but rather accentuating it. Although HBD6 expression is the likely source for these events, specific interaction between
adenovirus vector and HBD6 expression cannot be ruled out. While HBD6 efficacy against pathogenic organisms has not been established, this study did show the first in vivo recruitment of neutrophils by beta-defensin expression and that HBD6 expression during gene therapy has biological activity in the neonatal lamb lung.

Adenovirus mediated gene therapy in the lung has been hindered by the inflammatory response it generates. In this study a high concentration of adenovirus (23, 26) was used for transfection, which accentuated PIV3 activity. Recent technological advances such as pretreatment of airways with sodium caprate or complexing the adenovirus with DEAE dextran have been used to provide means of reducing the adenoviral titer required and thus minimizing the adverse effects (12). Another limitation of this study is the small number of lambs used in each group, a common problem associated with large animal work, which sometimes makes statistical significance challenging due to inter animal variations (24). However, large animal studies (as in this case) can provide novel insights to disease mechanisms not readily observed in rodent or other laboratory animal models (14). In this study we used an ovine isolate of PIV3 virus for the neonatal lamb study which is comparable to human PIV3 in disease and lesions (7, 15, 20). This is the first study to show high dose adenoviral gene therapy may exacerbate a concurrent PIV3 infection, a concern that needs to be further addressed for future adenovirus gene therapy. Additionally, this is the first study to show in vivo HBD6 expression has biological activity, specifically in neutrophil recruitment with mild aggravation of concurrent PIV3 infection.
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Figures

Figure 1. Body temperature for the treatment groups. The PIV3 group had significantly increased temperatures over the control group on day 6 while the PIV3/Ad/HBD6 group had increased temperatures over controls on days 2, 5, 6 and 7, (*p<0.01 or **p<0.05 versus control).

Figure 2. Respiration rate for treatment groups. On day 2 the PIV3/Ad group and the PIV3/Ad/HBD6 groups (p=0.08) had increased respiration rate compared to the PIV3 group. Starting at day 4 the PIV3 infected groups had increased respiration rates over controls with the PIV3/Ad/HBD6 group being slightly higher on days 6 and 7, (*p<0.05 versus control; **p<0.05 versus PIV3 group).
Figure 3. Daily milk consumption for the treatment groups. The PIV3 infected groups all had similar decreases in milk consumption, (*p<0.05 versus control).

Figure 4. Average daily gain for the treatment groups. The PIV3 and PIV3/Ad/HBD6 groups had a significant decrease in growth while the PIV3/Ad group had a slightly decreased growth rate compared to controls, (*p<0.05 versus control).
Figure 5. Pulmonary neutrophil density in the treatment groups. All the PIV3 infected groups had higher neutrophil density than did the control group. The PIV3/Ad/HBD6 group had increased neutrophil density over PIV3 and PIV3/Ad groups, (*p<0.05 versus control; **p<0.05 versus PIV3 or PIV3/Ad groups).
Figure 6. Immunohistochemistry for PIV3 in the A) PIV3 group, B) PIV3/Ad group and C) PIV3/Ad/HBD6 group, (all 400x magnification). The PIV3/Ad and PIV3/Ad/HBD6 groups had increased cellular staining over the PIV3 group. The control group had no staining (not shown).
Figure 7. Cellular PIV3 antigen staining in treatment groups. PIV3/Ad and PIV3/Ad/HBD6 groups each had significant increases in PIV3 antigen staining over the PIV3 group. The control group had no staining, (*p<0.05 versus PIV3 group).

Figure 8. Mitotic rate in the treatment groups. PIV3, PIV3/Ad and PIV3/Ad/HBD6 groups had similar significant increases in mitotic rate over the control group with no significant differences among the PIV3 infected groups, (*p<0.05 versus control group).
Figure 9. Syncytia cell formation in the treatment groups. The combined PIV3/Ad and PIV3/Ad/HBD6 had significantly higher syncytia cell formation than did the PIV3 group, (*p<0.05 versus PIV3 group).

Figure 10. SP-D mRNA expression in the treatment groups. PIV3, PIV3/Ad and PIV3/Ad/HBD6 groups are all increased compared to the control group with moderate increase in the PIV3/Ad/HBD6 group over the PIV3/Ad group, (*p<0.05 versus control group).
Figure 11. SP-A mRNA expression in the treatment groups. The combined PIV3/Ad and PIV3/Ad/HBD6 groups had significantly increased SP-A mRNA compared to the PIV3 group, (*p<0.05 versus PIV3 group).

Figure 12. SBD1 mRNA expression in the treatment groups. The combined PIV3/Ad and PIV3/Ad/HBD6 groups had significantly increased SBD1 mRNA compared to the PIV3 group, (*p<0.05 versus PIV3 group).
CHAPTER 5. GENERAL CONCLUSION

Death in preterm infants is often attributed to respiratory disease and this same age group is at increased risk for severe Paramyxoviral disease (2, 18). Because the respiratory epithelia are not fully developed at this time, suboptimal expression of innate immune factors, in part, is thought to predispose high risk groups to infection (5, 12, 24). Supplementation of innate immune factor such as beta-defensins could augment the status of innate pulmonary immunity. The preterm/perinatal lamb is a viable model for the study of Paramyxoviral infection, beta-defensin expression, and adenoviral gene therapy. This work 1) characterizes RSV infection in the preterm lamb model, 2) defines the developmental distribution and expression of SBD2 and 3) describes the influence HBD6 expression by adenoviral mediated gene therapy on the clinicopathological features of PIV3 infection.

RSV in the Preterm Lamb Model

There is currently no distinct RSV animal model which can be used to study the predisposition to age-dependent disease severity seen in preterm infants. Work in this dissertation demonstrates that preterm lambs are susceptible to bovine RSV infection and is a recognized viral model that parallels human RSV infection (9, 11). Infected lambs exhibited clinical signs and lesions comparable with that of human RSV disease. The clinical features were similar to or slightly less severe than that of human infection and the gross and microscopic lesions were consistent to that described for severe infection in preterm infants (8). The preterm lamb RSV model
has several advantages compared to other RSV models (e.g. rodents) including similar lung development and susceptibility to natural RSV infection (11, 16).

In addition, preterm RSV infection was compared to neonatal lambs given similar or three-logarithm higher TCID₅₀ inoculum. The cellular antigen staining and syncytial cell formation were both significantly increased in the preterm group. This suggests that the preterm lambs were deficient in their ability to clear RSV infection compared to either group of neonatal lambs, even the one given a higher titer viral inoculum.

This work demonstrated that preterm lambs are susceptible to RSV infection, a novel approach to using the full-term lamb model of RSV infection. More importantly, this work also showed reduced RSV clearance in preterm versus the full-term lambs with similar or high inoculum virus. Age-dependent RSV clearance in preterm lambs was similar to that seen in cotton rats used as a model of susceptibility to severe RSV infection in elderly humans (3). This inefficient RSV clearance is important as it can propagate detrimental airway inflammation and possibly be associated with RSV persistent infection or latency (15, 17, 21).

Sheep Beta-Defensin-2 Ontogeny

A putative factor in the susceptibility of preterm infants to severe Paramyxoviral infection is the immature expression of the innate antimicrobial factors. In the sheep model, SBD2 has been suggested to have developmental regulation in the gastrointestinal tract, but the extent and distribution of this expression in the lung and other tissues was not previously defined (10).
Tissues were collected from late gestational fetuses, neonates, and adult sheep in order to define the tissue distribution and expression of SBD2. The SBD2 mRNA and peptide were predominantly found in the gastrointestinal tract with highest expression in the jejunum and decreasing to the rectum. There was a progressive increase in the level of SBD2 expression in the gastrointestinal tissues with age reaffirming the concept of developmental regulation. SBD2 did not show consistent pulmonary expression in adult sheep. Preliminary work by others in our lab confirmed the lack of pulmonary expression and suggested SBD2 was not inducible in the lung.

The SBD2 tissue distribution was wider in the developing younger animals compared to the mature adult sheep. This could suggest SBD2 has a developmental function in cellular proliferation or differentiation as has been described for other beta-defensins (1, 4, 14).

**Adenoviral Mediated Beta-Defensin Expression**

As mentioned above, there is a putative role of "immature" epithelial expression of beta-defensins as a predisposing factor for severe Paramyxoviral infection. A potential avenue for prophylactic or therapeutic intervention could include enhanced expression of a beta-defensin. Gene therapy would be an appropriate vehicle to accomplish this task since exogenous regulators of innate beta-defensin expression are not well-defined (20). HBD6 was selected as it was a recently described human beta-defensin with no concurrent expression in sheep and since it was available in an adenoviral vector.
In this study, *in vivo* HBD6 expression caused significant increases in the pulmonary recruitment of neutrophils with slightly enhanced severity of clinicopathological parameters of infection. Instead of reducing PIV3 inflammation and disease, adenoviral-mediated HBD6 expression enhanced it. This study is the first to show *in vivo* biological activity of HBD6. Unexpectedly, this study suggests that enhanced expression of HBD6 is detrimental during PIV3 infection and would likely complicate the case management of affected patients rather than help them. The adenovirus vector and the unregulated HBD6 expression are possible contributing factors to the enhanced PIV3 severity in this study and need to be better defined before making judgment on the potential of beta-defensin therapy.

As indicated, the use of adenovirus vectors, regardless of gene insert, was also detrimental to clinical and lesion parameters. Adenoviral vectors have been associated with inflammation, in part, due to the deletion of regions in the viral genome (e.g. E1) that are associated with host immunosuppression (19). Additionally, the high dose of adenovirus used in the study likely contributed to the enhanced inflammation (6). We were surprised to find that the presence of adenoviruses causes enhanced PIV3 activity in the neonatal lungs. This novel finding is significant as pulmonary adenoviral gene therapy research often involves subjects that are predisposed to Paramyxoviral infection. For example, in cystic fibrosis patients, concern over infection are mostly limited to bacteria such as *Pseudomonas aeruginosa*, but our works emphasizes that viral infection should also be a concern especially when performing adenoviral gene therapy. Our findings emphasize 1) the perinatal lamb as a viable PIV3 model providing novel insights to
pulmonary disease, 2) the importance of viral infection in these high risk individuals and 3) that gene therapy should warrant a high level of caution in its potential for this and other adverse effects.

**Recommendation for Future Efforts**

The preterm lamb model needs to be fully utilized for further efforts on Paramyxoviral infection given the novel age-dependent differences seen with RSV infection. For comparison, future work should include in addition to neonatal lambs, younger preterm lambs (~day 125-130) that require mechanical ventilation and preterm lambs (Caesarian-section - day 139) that are not infected until they are the same conceptional age as full term neonatal lambs. This would help define the developmental aspects of the susceptibility. Additional factors to evaluate in future work should include pulmonary function tests and antimicrobial peptide/protein expression (SP-A, SP-D, and SBD1). Also, I would highly recommend additional studies to further define the pathogenesis of bovine RSV by various inoculation routes and subsequent time course for lesion development. Having a well-defined RSV inoculation model with established replication and time courses for lesion development would be helpful for characterizing the potential value of novel prophylactic and therapeutic therapies.

Concerning SBD2, I think potential for pulmonary expression need to be further addressed *in vitro*, specifically whether SBD2 expression is inducible. While preliminary efforts by others in our lab suggest it is not inducible, further *in vitro* efforts using various potential inducers (growth factors, cytokines, pathogen
products, etc.) to assure it is not a factor in pulmonary innate immunity. If in the end SBD2 does not show evidence of pulmonary involvement then beta-defensin pulmonary research can focus on SBD1 (7). Concerning SBD2's extra pulmonary involvement, I would suspect that its robust expression would provide a great model for inflammatory conditions of the intestines specifically Crohn's disease, Johne's disease, infectious diseases and inflammatory bowel disease (22, 23). There is great interest in the defensins of the gut related to these conditions, whether they are involved in the pathogenesis of the lesions or as possible therapeutic agents.

Our work with the adenoviral vectors need to be further addressed, specifically, define the mechanism for the enhanced PIV3 disease associated with adenoviral mediated therapy. I would start by using an in vitro culture model to try and rule out direct adenovirus and PIV3 interaction. This will likely need to be pursued in vivo as I speculate the mechanism(s) are related to the enhanced inflammatory response thus either changing cellular signaling for the advantage of the virus or by enhancing exposure to additional viral entry sites (e.g. basolateral membrane). Additionally, future efforts with the adenovirus vector need to minimize the adenovirus-associated inflammation. One way this could be accomplished in the lung is by pretreatment with modifying agents (e.g. sodium caprate) to expose virus binding sites and enhanced transfection with a much smaller dose of vector (6). Another option would be to go to a third or fourth generation adenovirus vector which have decreased inflammatory responses compared with first or second generation vectors (13). This could decrease the adenovirus specific inflammatory response, an adverse consequence in the lung.
The enhanced neutrophilic recruitment along with clinical signs and inflammation seen with adenoviral mediated HBD6 expression are important concerns and may dampen the enthusiasm of some to beta-defensin therapy. However, the potential contributing factors such as the adenoviral-vector delivery mechanism and the unregulated expression of HBD6 need to be considered in future efforts to define the therapeutic potential of the beta-defensin for pulmonary use. The mechanism of the delivery (adenovirus vector) could be replaced by direct placement of a known amount of recombinant pro-peptide beta-defensin into the lung by tracheal instillation or nebulization. Additionally, as the regulation of epithelial differentiation and/or beta-defensin expression are further defined these potential compounds (e.g. retinoic acids, glucocorticoids, growth factors, etc.) could be used to up regulate expression. These could control the amount of beta-defensin expression in the lung and minimize the potential of harmful events.

References


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