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Abstract

Vascular endothelial growth factor (VEGF) is increasingly recognized as a perinatal regulator of lung maturation and surfactant protein expression. Preterm and young infants are at increased risk for pulmonary immaturity characterized by insufficient surfactant production as well as increased risk for severe manifestations of respiratory syncytial virus (RSV) infection. Innate immune components including surfactant proteins A and D, and β-defensins have putative antimicrobial activity against pulmonary pathogens including RSV. Our hypothesis was that recombinant human VEGF (rhVEGF) pretreatment therapy would decrease RSV disease in the perinatal lamb RSV model. Newborn lambs were pretreated with rhVEGF, betamethasone, or saline and then inoculated with bovine RSV or sterile medium. Tissues were collected 5 d postinoculation, corresponding to the initiation of severe lesions and peak viral replication. In RSV-infected lambs, rhVEGF therapy increased the mean daily body temperature, decreased airway neutrophil exudate, and reduced RSV replication compared with betamethasone or saline pretreatment. Furthermore, rhVEGF therapy significantly mitigated the RSV-induced increase in surfactant protein A mRNA expression and decrease in surfactant protein D mRNA expression. In control (non-RSV-infected) lambs, pretreatment with rhVEGF increased sheep β-defensin-1 (SBD1) mRNA expression, but no alteration in surfactant proteins A and D was detected. This novel study demonstrates that rhVEGF pretreatment mitigates RSV disease and, in addition, rhVEGF regulation of innate immune genes is dependent on RSV infection status.

Disciplines
Veterinary Pathology and Pathobiology

Comments

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ABSTRACT

Vascular endothelial growth factor (VEGF) is increasingly recognized as a perinatal regulator of lung maturation and surfactant protein expression. Preterm and young infants are at increased risk for pulmonary immaturity characterized by insufficient surfactant production as well as increased risk for severe manifestations of respiratory syncytial virus (RSV) infection. Innate immune components including surfactant proteins A and D, and β-defensins have putative antimicrobial activity against pulmonary pathogens including RSV. Our hypothesis was that recombinant human VEGF (rhVEGF) pretreatment therapy would decrease RSV disease in the perinatal lamb RSV model. Newborn lambs were pretreated with rhVEGF, betamethasone, or saline and then inoculated with bovine RSV or sterile medium. Tissues were collected 5 d postinoculation, corresponding to the initiation of severe lesions and peak viral replication. In RSV-infected lambs, rhVEGF therapy increased the mean daily body temperature, decreased airway neutrophil exudate, and reduced RSV replication compared with betamethasone or saline pretreatment. Furthermore, rhVEGF therapy significantly mitigated the RSV-induced increase in surfactant protein A mRNA expression and decrease in surfactant protein D mRNA expression. In control (non-RSV-infected) lambs, pretreatment with rhVEGF increased sheep β-defensin-1 (SBD1) mRNA expression, but no alteration in surfactant proteins A and D was detected. This novel study demonstrates that rhVEGF pretreatment mitigates RSV disease and, in addition, rhVEGF regulation of innate immune genes is dependent on RSV infection status.

INTRODUCTION

RESPIRATORY SYNCYTIAL VIRUS (RSV) is an enveloped, negative sense, single-stranded RNA pneumovirus that is a significant cause of seasonal respiratory disease in humans (18). In healthy adults, RSV infection often results in a self-limiting respiratory disease. Yet, certain subpopulations including preterm and low birth weight infants are at increased risk for severe manifestations of RSV disease requiring hospitalization (24,32,40). RSV is

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the leading cause of bronchiolitis in infants and during the 1980s and into the mid-1990s annual RSV-associated hospitalization in the United States was estimated to be over 125,000 with nearly 500 deaths (29,35,36). Lesions frequently associated with severe RSV disease include necrotizing bronchiolitis (causing atelectasis, hyperinflation, and wheezing) and pneumonia (characterized radiographically as interstitial infiltrates, alveolar filling, and consolidation) (18). Bovine RSV infection in the perinatal lamb model is a viable model for study of the RSV disease and innate immune response (20,25).

Preterm and young infants are also predisposed to developing respiratory distress syndrome (RDS), which is associated with immature development of pulmonary surfactant (33). Glucocorticoid therapy, a promoter of lung maturation that was originally developed using sheep models, is conventionally used as a prophylactic treatment for premature birth (5,31,39). Unfortunately, glucocorticoid administration is also associated with potential short- to long-term adverse effects including alterations in neurological and cognitive development (21). The potential for adverse effects by glucocorticoids makes the search for novel and safer surfactant regulators a priority. Human airway epithelial explants treated with vascular endothelial growth factor (VEGF) exhibited proliferation with increased surfactant mRNA and protein expression (6) and surfactant proteins A and D have antiviral activity including opsonization and aggregation of RSV, and activation of macrophages (16). Furthermore, preterm mice treated with exogenous recombinant human VEGF (rhVEGF) had increased pulmonary maturation and survival (8). VEGF therapy has been constrained by concerns about potential adverse effects. For instance, transgenic mice with constitutive chronic expression of VEGF in respiratory epithelium had pulmonary lesions including chronic hemorrhage and alveolar remodeling (22). In a sheep model, intrabronchial deposition of exogenous rhVEGF induced a dose-dependent recruitment of monocyte/macrophages into the lung, causing gross and microscopic lesions (27). Although safety concerns regarding VEGF therapy are legitimate, studies of short-term and lower dose applications thus far have reported minimal clinical complications (8,27).

The prospect of rhVEGF therapy in perinatal patients at risk for RDS could have additional ramifications as these same patients are at elevated risk for severe RSV disease. Preterm infants have reduced surfactant protein expression including surfactant proteins A and D, which both have proven anti-RSV activity (15,17). Because VEGF is suggested to induce lung maturation and surfactant protein expression, this putative increase in (antiviral) surfactant proteins might prove useful as a therapy to prevent severe RSV disease (6,8,15). The hypothesis of this study was that rhVEGF pretreatment would diminish RSV disease in perinatal lambs. We compared this novel RDS therapy with traditional glucocorticoid therapy (betamethasone) and sham (sterile medium) treatment. In this study we characterize RSV infection through clinical signs, lesions, morphometry, and innate immune gene expression.

MATERIALS AND METHODS

Animals

Date-mated pregnant ewes were obtained from Laboratory Animal Resources (Iowa State University, Ames, IA), with all procedures approved by the Animal Care and Use Committee. After natural parturition, neonatal lambs (6–12 h old) were randomly pretreated with sterile saline (20 mL, intratracheal injection), sterile saline (20 mL, intratracheal) plus betamethasone (4.0 mg/kg, intramuscular), or recombinant human vascular endothelial growth factor (rhVEGF, 5 μg/mL $\times$ 20 mL, intratracheal; Invitrogen, Carlsbad, CA) (Table 1). After 30 min for acclimation, the lambs were further divided into two treatment groups receiving either sterile saline (20 mL, intratracheal) or bovine respiratory syncytial virus (bovine RSV strain 375, 10^3 to 10^4 TCID$_{50}$ [50% tissue culture infective doses]/mL $\times$ 20 mL, intratracheal). Lambs were given daily antibiotic (ceftiofur, 2.2 mg/kg per day, intramuscular) to prevent bacterial complications (41). During the course of infection, lambs were monitored for clinical signs including body temperature. From our previous experience with this model of RSV disease, severe lesion development and peak viral replication occur on day 5 of infection (23,25). Lambs were killed with

TABLE 1. EXPERIMENTAL DESIGN FOR ASSESSING VEGF PRETREATMENT IN PERINATAL RSV DISEASE[a]

Group	Pretreatment[b]	Treatment[c]
C/Media	Sterile saline	Sterile medium
V/Media	VEGF	Sterile medium
B/Media	Betamethasone	Sterile medium
C/RSV	Sterile saline	Bovine RSV
V/RSV	VEGF	Bovine RSV
B/RSV	Betamethasone	Bovine RSV

[a]Lambs were monitored during infection for clinical signs and tissues were collected on day 5 of infection to assess for lesions, morphometry, immunohistochemistry, and gene expression (RSV, SBD1, SP-A, and SP-D).

[b]Intratracheal injection (20 mL, sterile saline or VEGF [5 μg/mL]) or intramuscular injection (betamethasone, 4 mg/kg) was administered approximately 30 min before treatment.

[c]Intratracheal injection (20 mL) of sterile medium or RSV (bovine RSV strain 375, 10^3 to 10^4 TCID$_{50}$/mL, intratracheal).

sodium pentobarbital on day 5 of infection, and lungs were examined for gross lesions. Tissue was collected bilaterally from the cranial and middle lobes. Tissues were either snap frozen on dry ice for quantitative polymerase chain reaction (qPCR) or positioned in cassettes and placed in 10% neutral-buffered formalin (24–48 h) for morphologic and immunohistochemical analysis.

RNA isolation

Total tissue RNA was isolated from whole lung tissue (right middle lobe) for gene expression analysis by hydrolysis probe-based fluorogenic one-step real-time qPCR. Briefly, lung tissue samples were weighed, placed into 3 mL of TRIzol reagent (Invitrogen), and homogenized. The homogenate was vortexed and nuclease-free chloroform (200 μL) was added to each sample, mixed, and then microcentrifuged at $12,000 \times g$ for 10 min. Top aqueous layers were transferred into 500 μL of nuclease-free 2-propanol (Fisher Chemical, Fairlawn, NJ), vortexed, allowed to sit and again microcentrifuged with subsequent removal of the top aqueous layer. The remaining pellet was washed (75% nuclease-free ethanol) and microcentrifuged, the final supernatant was removed, and the remaining samples were allowed to air dry under a fume hood. Each pellet was resuspended (nuclease-free 0.1 mM EDTA, pH 7.0), heated to 65°C for 5 min, and stored at 4°C. RNA isolates were assessed at 1:50 dilution for quantity and purity by spectrophotometry at 260 and 280 nm followed immediately by DNase treatment with TURBO DNase (TURBO DNA-free kit; Ambion, Austin, TX). For each sample, 80 μL of each supernatant RNA was recovered and diluted 1:10 with nuclease-free water (Ambion), resulting in 800 μL of each RNA isolate.

qPCR

qPCR was carried out as a one-step process as previously described in detail (14). Each of our final 25-μL one-step real-time qPCRs contained the following: 12.5 μL of one-step master mix (TaqMan one-step RT-PCR master mix reagents kit; Applied Biosystems, Foster City, CA), MultiScribe reverse transcriptase (RT, 0.25 U/μL), RNase inhibitor (0.4 U/μL), optimal forward and reverse primer and fluorogenic probe concentrations (Table 2), nuclease-free water, and 6.5 μL of each RNA sample/template (14,16). Thermocycling conditions for all qPCRs were as follows: 35 min at 48°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 58°C. All plates were run in duplicate on a GeneAmp 5700 sequence detection system (Applied Biosystems) and all output data were processed as custom Excel files (Microsoft, Redmond, WA). The Pfaffl equation (value = $[(E_{\text{target}})^{\Delta Ct(\text{control} - \text{treated})}]/[(E_{\text{housekeeper}})^{\Delta Ct(\text{control} - \text{treated})}]$) was used for relative quantitation based on target-specific fluorescent signals

TABLE 2.   PRIMERS AND PROBES FOR OVINE GENE EXPRESSION ASSESSED BY REAL-TIME qPCR

Target gene	Primer (concentration)	Sequence[a]
SBD1	Fwd (1000 nM)	5′-CCATAGGAATAAAGGCGTCTGTG
	Rev (1000 nM)	5′-CGCGACAGGTGCCAATCT
	Probe (150 nM)	5′-6FAM-CCGAGCAGGTGCCCTAGACACATGA-TAMRA
SP-A	Fwd (500 nM)	5′-TGACCCTTATGCTCCTCTGGAT
	Rev (500 nM)	5′-GGGCTTCCAAGACAAACTTCCT
	Probe (50 nM)	5′-6FAM-TGGCTTCTGGCCTCGAGTGCG-TAMRA
SP-D	Fwd (500 nM)	5′-ACGTTCTGCAGCTGAGAAT
	Rev (500 nM)	5′-TCGGTCATGCTCAGGAAAGC
	Probe (100 nM)	5′-6FAM-TTGACTCAGCTGGCCACAGCCCAGAACA-TAMRA
Bovine RSV Ncap	Fwd (1000 nM)	5′-CAGTCAAGAATATTATGCTTGGTCATG
	Rev (1000 nM)	5′-CCTAACTTTTGTGCATATTCATAGACTTC
	Probe (150 nM)	5′-6FAM-CAACCTGTTCCATTTCTGCTTGTACGCTG-TAMRA
ovRPS15	Fwd (1000 nM)	5′-CGAGATGGTGGGCAGCAT
	Rev (1000 nM)	5′-GCTTGATTTCCACCTGGTTGA
	Probe (150 nM)	5′-VIC-CCGGCGTCTACAACGGCAAGACC-TAMRA
hRibo18S	Fwd (50 nM)	5′-CGGCTACCACATCCAAGGAA
	Rev (50 nM)	5′-GCTGGAATTACCGCGGCT
	Probe (200 nM)	5′-VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA

[a]6FAM or VIC, 5′ fluorescent reporter dye; TAMRA, fluorescent quencher dye.

generated during qPCR of total RNA from the whole lung homogenates in this study (30). For each animal, RNA sample real-time target gene amplifications were normalized to the geometric mean of two housekeeping genes, hRibo18S and ovRPS15 (the latter sequence was kindly provided by S. Limesand, University of Colorado Health Sciences Center, Aurora, CO).

Morphometry

Gross lesions were assessed on the basis of a scoring system in which multifocal to coalescing areas of plum-red consolidation were expressed as a percentage of lung area: 0, 0%; 1, <5%; 2, 5–20%; 3, 21–40%; 4, 41–60%; and 5, >60%.

Neutrophil infiltration into bronchioles was assessed by a pathologist (26). Briefly, low-magnification sites ($n = 10$) were randomly selected, a bronchiole from each site was targeted, and the number of neutrophils within the basement membrane and lumen were counted and values per section averaged.

Statistical analysis

In pretreatment of qPCR data, we found that the standard deviation of replicated measurements increased with its respective mean. Thus all qPCR measurements were log transformed in order to stabilize variance.

For each RNA sample, the arithmetic mean of log transformations of all measurements of its housekeeper genes (two genes) was used as a normalization factor. This normalization factor was then subtracted from each measurement of the target gene after being log transformed. The result is the relative gene expression of each target gene. This operation is equivalent to dividing raw measurements of each target gene by the geometric means of all measurements of its corresponding housekeeper genes.

We used a standard two-sample t test to compare expression of the same target gene under different treatment conditions. In the case of treatments with significantly different variances, we compared means through a Welch modified two-sample t test. Variances between different treatments were compared through an F statistic.

We used a balanced two-way analysis of variance (seven replications per treatment) to test for effect of treatments on body temperature, but grouping for possible variability due to day postinfection. Seven replications were randomly chosen from treatments with more than seven replications. Mean body temperature was calculated by taking the sum of daily body temperatures for each animal during the course of the infection and dividing by the days assessed. Significant differences in body temperature means of different treatments was established through the least significant difference (LSD) method.

We used two-way analysis of variance to test the treatments on scores for gross lesions and neutrophil infiltration. If significance was detected as a result of treatment then post-hoc tests were applied to scientifically relevant comparisons.

RESULTS

Clinical RSV infection

During the course of infection, all the RSV-infected animals exhibited mild to moderate clinical signs including increased body temperature, tachypnea, and cough, whereas the control groups lacked clinical signs. No significant difference between treatments was detected in degree of clinical signs such as tachypnea, cough, appetite, and clinical appearance (data not shown). RSV treatment caused a significant increase in mean body temperature during the course of infection compared with sham inoculation with sterile media (C/RSV > C/Media; $p < 0.001$) (Fig. 1A). Pretreatment of sham-inoculated groups caused no significant alteration in mean temperature; however, rhVEGF pretreatment of RSV infection increased mean body temperature compared with betamethasone pretreatment (V/RSV > B/RSV; $p < 0.05$), RSV infection alone (V/RSV > C/RSV; $p < 0.05$), and rhVEGF pretreatment alone (V/RSV > V/Media; $p < 0.001$). The increased body temperature induced by rhVEGF pretreatment in the RSV groups was not characterized by extremely high values on any given day, but rather by persistently elevated values throughout the course of infection.

RSV lesions and replication

All RSV-infected groups exhibited the typical RSV-induced gross lesions of plum to red consolidation, whereas the group given media lacked lesions. Significant difference in distribution and severity of gross lesions between treatment groups was not detected (Fig. 1B). Microscopically, whereas all RSV-infected groups had typical RSV lesions (necrotizing bronchiolitis, epithelial syncytia, etc.), there was significant reduction in neutrophilic inflammation within the RSV-infected bronchioles of rhVEGF-pretreated versus saline ($p < 0.07$) and betamethasone groups ($p < 0.05$) (Fig. 1C).

qPCR analysis of whole lung homogenates detected a significant reduction in RSV mRNA expression in the rhVEGF pretreatment group compared with saline ($p < 0.00001$) and betamethasone ($p < 0.01$) (Fig. 2A). Analysis between mean body temperature during the course of infection and levels of RSV replication by day 5 of infection demonstrated a strong correlation among individual animals ($p < 0.06$) and a group interaction
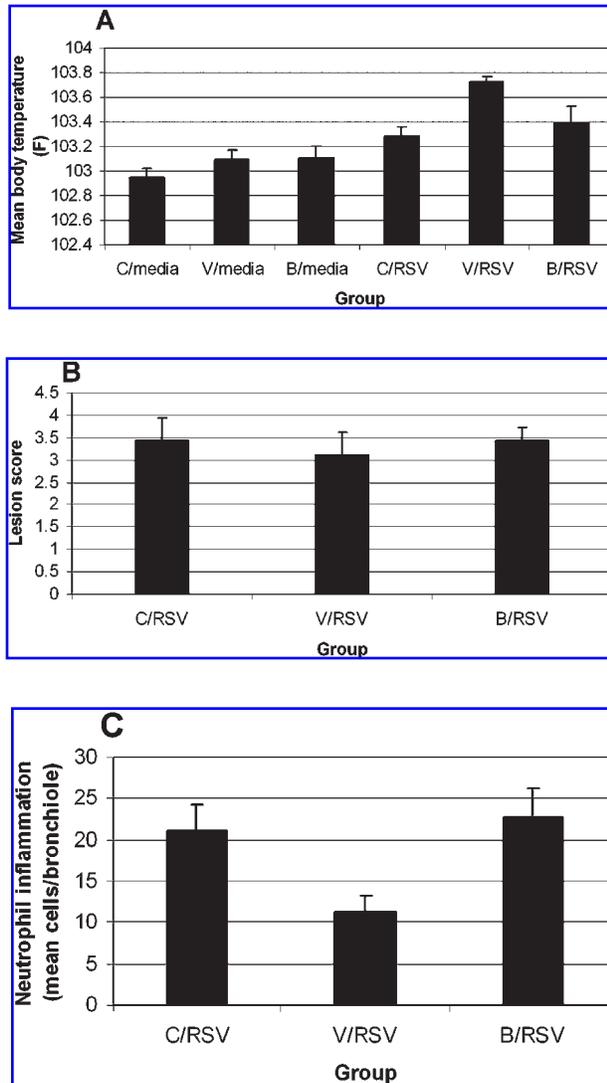
FIG. 1. Clinical comparison of sham- and RSV-infected neonatal lambs pretreated with saline, betamethasone, or rhVEGF. (**A**) Mean body temperature. RSV infection overall caused a significant increase in group mean body temperatures ($p < 0.001$). In RSV-infected lambs, rhVEGF pretreatment increased mean body temperature compared with RSV infection alone (C/RSV; $p < 0.05$), betamethasone (B/RSV; $p < 0.05$) pretreatment or compared with rhVEGF pretreatment of controls (7V/Media; $p < 0.001$). (**B**) Gross lesions. No significant alterations in gross lesions were detected. (**C**) Bronchiole inflammation. The number of neutrophils was reduced after rhVEGF pretreatment compared with saline (V/RSV < C/RSV; $p < 0.07$) or betamethasone (V/RSV < B/RSV; $p < 0.05$).

($r = -0.88$) can be seen in the group plot, demonstrating the V/RSV group as having the higher body temperature and lower viral replication than the other pretreatment groups (Fig. 2B).

Innate immune gene expression

Sheep β-defensin-1 (SBD1) mRNA expression was increased in rhVEGF versus saline or betamethasone pretreated, sham-infected groups ($p < 0.05$) (Fig. 3A). During RSV infection, the saline pretreatment group had increased SBD1 expression (C/RSV > C/Media; $p < 0.1$); however, rhVEGF pretreatment of RSV infection suppressed SBD1 expression compared with either the rhVEGF or RSV group control (V/RSV < V/Media or C/RSV; $p < 0.05$).

Surfactant protein D (SP-D) mRNA expression was not significantly altered by pretreatments in the sterile media controls (Fig. 3B). Independently, RSV infection reduced SP-D mRNA expression (C/Media > C/RSV; $p < 0.05$). This reduction in SP-D mRNA expression by RSV infection was mitigated to near control levels by rhVEGF and betamethasone pretreatment (C/RSV < B/RSV or V/RSV; $p < 0.05$).

SP-A expression, similar to SP-D expression, was statistically unaltered by pretreatment in the control groups (Fig. 3C). RSV infection increased SP-A expression
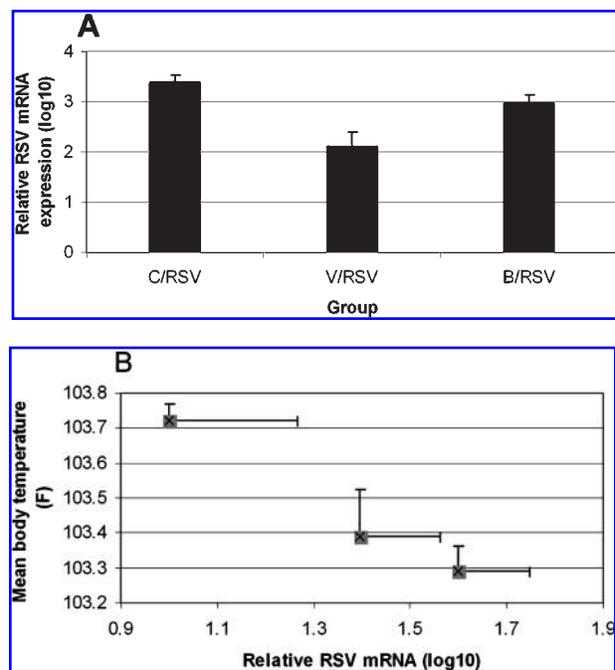

FIG. 2. RSV replication in neonatal lambs pretreated with saline, betamethasone, or rhVEGF. (**A**) RSV mRNA expression. RSV mRNA expression ($\log_{10}$) was reduced in the rhVEGF pretreatment group compared with saline ($p < 0.00001$) and betamethasone ($p < 0.01$) pretreatment. (**B**) Body temperature and RSV replication interaction. Among lambs there was a trend toward reduced viral replication with higher mean body temperature ($p < 0.06$). This correlation ($r = -0.88$) was distinct between the treatment groups V/RSV = 103.7; B/RSV = 103.4; C/RSV = 103.3.
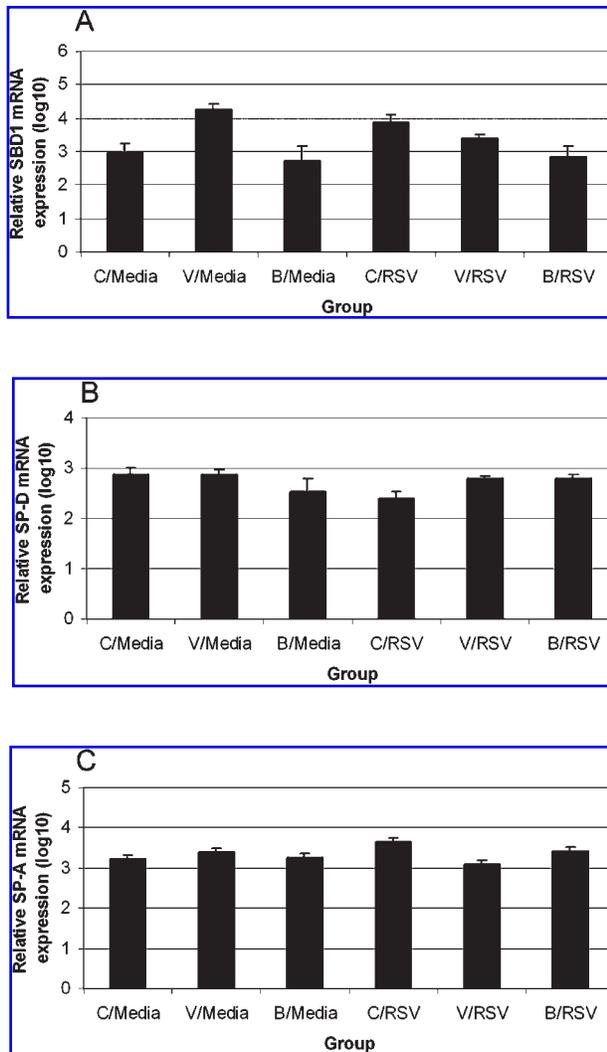
FIG. 3. Innate immune gene expression. (**A**) SBD1 mRNA expression ($\log_{10}$). SBD1 expression in sham inoculates was increased by rhVEGF relative to saline ($p < 0.05$) or betamethasone ($p < 0.05$) pretreatment, with similarly increased expression detected in RSV infection (C/RSV > C/Media; $p < 0.1$). Whereas rhVEGF pretreatment and RSV infection each increased SBD1 expression (V/Media > C/Media, $p < 0.05$; C/RSV > C/Media, $p < 0.05$), the combination of rhVEGF pretreatment and RSV infection suppressed SBD1 expression (V/Media vs. V/RSV, $p < 0.05$; C/RSV vs. V/RSV, $p < 0.05$). (**B**) SP-D mRNA expression ($\log_{10}$). SP-D expression was not significantly altered by pretreatment in sham-inoculated groups. RSV infection (C/Media > C/RSV; $p < 0.05$) reduced SP-D expression, whereas pretreatment with rhVEGF and betamethasone suppressed the RSV-induced alteration to near control levels (C/RSV < V/RSV or B/RSV; $p < 0.05$). (**C**) SP-A mRNA expression ($\log_{10}$). SP-A expression was unaltered by pretreatment in sham-inoculated groups. RSV infection increased SP-A compared with sham-infected control (C/RSV > C/Media; $p = 0.01$) and this RSV-induced alteration was suppressed by rhVEGF and betamethasone pretreatment (C/RSV > V/RSV or B/RSV; $p < 0.05$).

(C/RSV > C/Media; $p < 0.01$) but pretreatment with rhVEGF or betamethasone suppressed the RSV-induced alteration (C/RSV > V/RSV or B/RSV; $p < 0.05$)

DISCUSSION

The purpose of this study was to compare novel (rhVEGF), traditional (betamethasone), or sham (sterile medium) RDS therapy in a perinatal lamb model of RSV infection. The rationale for "pretreatment" of rhVEGF therapy was made on the presumption that rhVEGF could be used for RDS therapy (and increased antiviral surfactant proteins) and thereby would already be active in high-risk infants that become exposed to RSV. Indeed, rhVEGF pretreatment in this study mitigated several key parameters of RSV infection.

All RSV-infected lambs had mild to moderate clinical signs including increased body temperature, cough, and tachypnea consistent with previous work in our laboratory (23,25). Interestingly, clinical signs of RSV infection were not significantly mitigated except for body temperatures. In this particular animal model there is typically a transient, mild increase in body temperature on the first day because of inoculum (RSV antigen) delivery followed by another moderate increase on days 3–5 of infection corresponding to pulmonary viral replication and activation of the immune response. In this current study, rhVEGF pretreatment increased daily body temperature during the course of infection, resulting in an increase in mean body temperature. Interestingly, the rhVEGF pretreatment group also had decreased RSV viral mRNA expression on day 5 of the experiment, representing the time of maximum viral replication in this model (23,25). The increased mean body temperature in the rhVEGF pretreatment group likely affected RSV replication as they were inversely correlated. It is recognized that elevated body temperature is a byproduct of the innate immune system, often resulting from cytokine (e.g., interleukin [IL]-1, IL-6, and tumor necrosis factor [TNF]-α) expression (10). Increased temperature can inhibit *in vitro* replication of some viruses including influenza and feline immunodeficiency virus and temperature regulation is in part the foundational precept of RSV temperature-sensitive vaccines (2,7,34). The correlation of increased body temperature during the course of disease to decreased RSV replication is a novel finding for this perinatal RSV model.

We speculate that at least three different mechanisms may be involved in the sustained elevated body temperature and reduced viral expression by rhVEGF pretreatment. rhVEGF can interact with the endothelial VEGF receptor and promote angiogenesis, a prominent component of this process being enhanced vascular permeability (11,12). This subtle (not detected clinically) vascular

leakage could allow extravasation of antibodies or other innate immune components (e.g., mannose-binding protein) for antigen–antibody complex formation; an important source of pyrogenicity in viral infection (19). A second potential mechanism is related to the recruitment of monocytes and macrophages to sites of VEGF expression/administration (3,27). Monocytes/ macrophages are important producers of pyrogenic cytokines after phagocytosis of antigen (38). rhVEGF recruitment of monocytes/macrophages to the lung could contribute to enhanced opportunity for interaction with virus from the inoculum (early infection) or infected cells (mid to late infection) for cytokine production. Last, although body temperature was correlated with reduced viral inhibition, we cannot exclude the premise that increased temperature and reduced RSV replication are both mediated as a direct result of some innate mediator such as cytokine or cellular effects, such as B cell proliferation seen in mice (13,28). Specifically, alterations to β-defensin and surfactant protein expression, which both have both antiviral and immunomodulatory capacity, may have contributed to the reduced RSV replication.

In this model of RSV disease, neutrophil inflammation in bronchioles was reduced by rhVEGF pretreatment. Neutrophilic exocytosis is a major cause of epithelial damage and airway obstruction contributing to ventilation compromise during RSV disease in children (37,42). VEGF interaction with endothelium can regulate adhesion molecule expression and even enhance neutrophil emigration; however, in this case the reduced neutrophil recruitment was likely due to diminished virus replication as this is a significant regulator of neutrophil emigration in RSV disease (4,22,43).

Concerning innate immune gene mRNA levels, we detected differential regulation of innate immune genes by rhVEGF pretreatment and RSV infection. SBD1 mRNA expression was upregulated by rhVEGF pretreatment but not significantly altered during RSV infection. This novel finding of rhVEGF-induced β-defensin mRNA expression is interesting and this relationship may be defined in part as β-defensins and VEGF have been reported to synergize in the recruitment of dendritic cells and in vasculogenesis of tumors (9). The lack of SBD1 mRNA alteration during RSV infection is consistent with investigation of laser capture microdissected bovine RSV-infected and noninfected epithelia in which SBD1 mRNA expression was not changed (20). Although SBD1 lacks NF-κB regulatory elements, the regulatory pathway of SBD1 is not yet fully defined (1).

In contrast to SBD1, surfactant proteins A and D were not altered by rhVEGF pretreatment in control (non-RSV-infected) lambs, but rhVEGF pretreatment (and be-

tamethasone) did mitigate the magnitude of surfactant protein mRNA alteration seen during RSV disease. Betamethasone is a glucocorticoid therapeutically given to perinatal infants to increase surfactant expression for prevention of respiratory distress syndrome (5,38). VEGF has been proposed as a novel therapeutic for lung maturation and surfactant expression in place of glucocorticoids, which have potential adverse effects (8,21). Because rhVEGF and not betamethasone pretreatment diminished select parameters of RSV disease (e.g., viral replication and bronchiolar neutrophilic exudate), this suggests that the mitigation of surfactant protein mRNA alteration alone may not fully explain the RSV disease mitigation and that other factors are more fundamental.

In summary, this study demonstrates that rhVEGF pretreatment therapy can diminish select parameters of RSV disease. Furthermore, rhVEGF pretreatment interacts with RSV disease status to cause differential regulation of innate immune gene expression. The role of these innate immune gene alterations in relation to rhVEGF therapy of RSV infection is not fully known. These foundational results warrant further investigation into the kinetics of perinatal VEGF therapy and RSV disease.

ACKNOWLEDGMENTS

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