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Exogenous administration of vascular endothelial growth factor prior to human respiratory syncytial virus a2 infection reduces pulmonary pathology in neonatal lambs and alters epithelial innate immune responses

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Abstract

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

Keywords

innate immunity; pulmonary pathology; respiratory syncytial virus; sheep defensin; surfactant protein; vascular endothelial growth factor

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

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developed, there is still a great need to find and develop other therapeutic targets to decrease virus-induced pathology.

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

VEGF-A is a member of the vascular endothelial growth factor family, which also includes VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor. The gene encoding VEGF-A is comprised of 8 exons that are alternatively spliced to yield multiple protein isoforms with varying activities [6, 10]. The isoforms include VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆. VEGF₁₆₅ is the predominant isoform and the most potent [11]. VEGF₁₂₁ is freely diffusible, whereas VEGF₁₈₉ and VEGF₂₀₆ are bound within the extracellular matrix. VEGF₁₆₅ is both diffusible and sequestered to the cell surface and extracellular matrix via its heparin binding moieties.

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

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

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

METHODS

Experimental procedure

Animal use and experimental procedures were approved by Iowa State University's Animal Care and Use Committee. Neonatal lambs (2 to 3 days of age) were randomly assigned to 3 main groups: 2-day ($n = 16$), 4-day ($n = 16$), and 6-day ($n = 24$), based on length of infection

period. Within each of these groups, animals were then assigned to treatment groups (Table 1). All lambs were pretreated with recombinant human VEGF₁₆₅ (30 µg/kg) (Invitrogen or GenWay Biotech) or phosphate-buffered saline (PBS). Animals in the day 2 and 4 groups were treated via intrabronchial administration while those in the day 6 group were treated via intratracheal administration. Thirty minutes following pretreatment, animals were inoculated with human RSV virus (10 mL, 3×10^6 plaque-forming units [PFU]/mL) or cell growth medium (10 mL). RSV (A2 strain) was propagated in HEp-2 cells (American Type Culture Collection [ATCC], Manassas, VA). The inoculum was a cleared supernatant from infected cells that underwent a centrifugation step to remove cellular debris. The virus was tittered via plaque assay on VERO cells according to standard protocols. Prior to and following inoculation, lambs were administered daily antibiotics (ceftiofur, 2.2 mg/kg, intramuscular) to prevent bacterial pulmonary complications. Lambs were monitored daily for clinical signs of respiratory disease (coughing and wheezing) and temperatures were recorded. Animals were sacrificed by sodium pentobarbital on days 2, 4, and 6 post inoculation.

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

Immunohistochemical analysis

Immunohistochemical staining was performed for RSV antigen (1:50, overnight incubation, goat anti-human polyclonal antibody; BioDesign/Meridian) and for proliferating cells using an antibody against the Ki-67 protein (1:100, overnight incubation, mouse anti-human monoclonal antibody; Dako). Briefly, paraffin-embedded lung sections were cut at 5 µm thickness onto positively charged slides. Slides were incubated at 58°C for 25 minutes, deparaffinized in xylene, rehydrated in graded ethanol washes, and washed in phosphate-buffered saline (PBS). For RSV immunohistochemistry, tissue sections were treated with Pronase E (Protease Type XIV from *Streptomyces griseus*; Sigma) for 12 minutes at 37°C. Nonspecific binding was blocked by incubation in 20% normal swine. For Ki-67 immunohistochemistry, tissue sections were boiled for 15 minutes in 0.01 M citric acid (pH 6.0). Following overnight incubation with the primary antibodies, the slides were rinsed and then incubated with an appropriate biotinylated secondary antibody at a concentration of 1:300. A peroxidase block with 3% peroxide was followed by incubation with peroxidase-conjugated streptavidin (BioGenex) for 45 minutes. After 3 PBS washes, the color was developed with Nova Red. The slides were then counterstained with Harris' hematoxylin, dehydrated, and cover-slipped.

Histology scoring

Histologic lesion scores were determined from 2 lung sections per RSV-infected lamb in each pretreatment group (e.g., VEGF versus PBS). Sections were scored by a pathologist blinded to the study groups to determine total alveolar consolidation and cellular infiltration. Alveolar consolidation was defined by reduced expansion of alveolar lumen due to alveolar septal infiltration of neutrophils, lymphocytes, plasma cells, and type II cell hypertrophy along with intraluminal accumulation of neutrophils, macrophages, and small amounts of cell debris. A total of 5 20× fields were examined per lung section and alveolar consolidation was scored as: 0 = no consolidation; 1 = 1–30% consolidation; 2 = 30–50% consolidation; 3 = 60–80% consolidation; 4 = >80% consolidation. Values were averaged per section and converted to a percentage value.

Two lung sections from all animals were examined to determine the number of Ki-67 immunoreactive cells. A total of 10 20× fields were examined per lung section and values were averaged.

One-step real-time qPCR

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

Statistical analysis

Statistical analyses were performed with commercially available statistics software (GraphPad Prism, GraphPad Software). Data were analyzed for normal distribution and log transformed when necessary. Comparisons were done using 1-way analysis of variance (ANOVA) followed by Tukey's posttest analysis. Histologic lesion score data were analyzed using a Student *t* test. Data are represented as means (SEM). *P* < .05 was considered to be significant unless otherwise noted.

RESULTS

Clinical data

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

Histology and immunohistochemistry

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

Histologic lesion scores were calculated for each treatment group based on alveolar consolidation and inflammatory cell infiltration (Figure 2).

Real-time qPCR

Effect of rhVEGF on RSV mRNA expression—RSV mRNA levels were measured by RT-PCR to determine the effect of rhVEGF pretreatment on RSV replication. Lambs pretreated with rhVEGF had significantly higher RSV mRNA levels at day 4 post infection than RSV-infected lambs pretreated with PBS. However at day 6, lambs pretreated with rhVEGF had a marked decrease in RSV mRNA levels as compared to lambs pretreated with PBS (Figure 3).

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

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

Effect of RSV infection and rhVEGF administration on inflammatory mediators—RSV infection induced the expression of tumor necrosis factor α (TNF α) and interleukin-8 (IL-8) at day 2 post infection in both pretreatment groups (Figures 6, Figure 7). Administration of rhVEGF increased TNF α expression at day 4 (Figure 6). There was no significant alteration in expression of IL-6 and interferon β (IFN β) by RSV infection or rhVEGF administration.

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

Effect of RSV infection and rhVEGF administration on pulmonary cellular proliferation—VEGF can reduce apoptosis of both endothelial cells and pulmonary epithelial cells [13, 26]. The effect of VEGF on the growth and proliferation of lung epithelial cells following injury is not well known. To test this, cellular proliferation was measured within the lung parenchyma by immunohistochemistry for Ki-67 antigen (Figure

9). Immunoreactive cells had intense, dark brown nuclear staining. Immunoreactive cells were present within bronchiolar epithelium and multifocally within the alveolar interstitium. At day 4, RSV-infected lambs pretreated with rhVEGF had increased cellular proliferation compared to control (Figure 10). There was no significant difference in RSV-infected lambs pretreated with PBS at this time point compared to PBS control. At day 6, there were significantly more Ki-67-immunoreactive cells in RSV-infected lambs pretreated with PBS compared to lambs pretreated with VEGF. In fact, RSV-infected lambs pretreated with rhVEGF had no increase in cellular proliferation over control at day 6.

DISCUSSION

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

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

β -Defensins are antimicrobial peptides that have important roles in both the innate and adaptive immune responses. The antiviral activity of β -defensins include direct antiviral activity, chemotactic activity for macrophages and immature dendritic cells, and induction of cytokine signaling by monocytes and epithelial cells [35]. RSV infection induced

expression of SBD-1 at 2 and 4 days in both pretreatment groups. Pretreatment with rhVEGF in non-RSV-infected lambs induced expression of SBD-1 at days 4 and 6. The induction of SBD-1 expression by VEGF has been documented in a previous study showing that VEGF modulates components of the innate immune response [27]. At day 6, SBD-1 levels in RSV-infected lambs pretreated with VEGF were much lower than lambs pretreated with PBS. The lower expression level in this group is similar to the decreased expression observed with SP-A. Again, the cause for the lower expression in this group is unknown, but may be related to a decrease in viral-induced epithelial responses.

A study investigating VEGF levels in human infants with RSV infection showed up-regulation of VEGF in the nasal fluid [36]. In many human studies nasal fluid and bronchoalveolar lavage (BAL) are the only means of measurement, as tissue samples are often difficult to obtain. In this study, endogenous VEGF expression was increased at day 2 but decreased at day 6. VEGF mRNA was measured in lung homogenates, which may give an indication of the overall effect of RSV-induced inflammation on VEGF expression. VEGF is secreted both basolaterally and apically by pulmonary epithelial cells. In addition, VEGF₁₈₉ and VEGF₂₀₆ are bound to the extracellular matrix within the lung tissue and cleaved to active fragments by plasmin upon extracellular matrix breakdown [4]. Nasal secretion and BAL measurements provide information as to the expression level in one location only. An example of the location differences in VEGF levels can be shown in a study using a neonatal pig lung injury model, which reported decreased VEGF mRNA expression and protein levels in lung tissue with increased protein levels in the BAL [37]. This poses the question as to the significance of altered expression at various locations and time points in infection.

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

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

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

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

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

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FIGURE 1.

Photomicrograph of lung from an RSV-infected lamb pretreated with PBS (**A**) and an RSV-infected lamb pretreated with VEGF (**C**). RSV-infected lambs pretreated with PBS had suppurative bronchiolitis with locally extensive alveolar consolidation and infiltration of lymphocytes and plasma cells. RSV-infected lambs pretreated with VEGF had decreased alveolar consolidation, bronchiolitis, and inflammatory cell infiltration. RSV antigen staining by immunohistochemistry demonstrated multifocal immunoreactive cells within the bronchiolar epithelium, airway lumen and interstitium (**B**). Lambs pretreated with VEGF had decreased antigen at day 6 post infection (**D**). Bar = 100 μ m.

FIGURE 2.

Pretreatment with exogenous rhVEGF decreases histologic consolidation score indicating a decreased in alveolar consolidation and inflammatory cell infiltration (* $P < .01$).

FIGURE 3.

At day 2, lambs pretreated with rhVEGF have significantly higher RSV mRNA levels (A). At day 4 the RSV mRNA levels in the rhVEGF-pretreated lambs are decreasing (B), with expression levels significantly lower than control-pretreated animals at 6 days post infection (C) (* $P < .05$; ** $P < .01$).

FIGURE 4.

Pretreatment with exogenous rhVEGF and inoculation with RSV alters expression of surfactant protein A. At day 2, there were no significant differences in SP-A expression (A). At day 4, administration of rhVEGF increased SP-A expression in non-RSV-infected lambs, as compared to pretreatment PBS. Inoculation with RSV increased SP-A in both control and rhVEGF-pretreated groups (B). At day 6, SP-A expression in rhVEGF-pretreated non-RSV-infected lambs remained increased. SP-A expression was decreased in RSV-infected lambs that were pretreated with rhVEGF compared to those that were pretreated with PBS (C) (* $P < .05$; ** $P < .01$; *** $P < .001$).

FIGURE 5.

Pretreatment with exogenous rhVEGF and inoculation with RSV alters expression levels of sheep β -defensin-1. At day 2, inoculation with RSV increased SBD-1 levels in both pretreatment groups; however, SBD-1 expression was higher in animals pretreated with rhVEGF (**A**). At day 4, administration of rhVEGF increased SBD-1 expression in non-RSV-infected lambs, as compared to control. SBD-1 expression in RSV-infected animals remained increased (**B**). At day 6, SBD-1 expression in RSV-infected lambs pretreated with rhVEGF was significantly decreased compared to RSV-infected lambs pretreated with PBS. SBD-1 expression remained high in non-RSV-infected lambs pretreated with rhVEGF (**C**) (* $P < .05$; ** $P < .01$; *** $P < .001$).

FIGURE 6.

Pretreatment with exogenous rhVEGF and inoculation with RSV alters expression levels of TNF α . At day 2, TNF α expression was significantly increased in RSV-infected lambs in both pretreatment groups (**A**). At day 4, TNF α expression increased in non-RSV-infected lambs pretreated with rhVEGF. Expression decreased from day 2 levels in RSV-infected lambs, with higher levels in lambs pretreated with rhVEGF (**B**) (* $P < .05$; ** $P < .01$; *** $P < .001$).

FIGURE 7.
RSV infection increases expression of IL-8 in both pretreatment groups (* $P < .05$).

FIGURE 8.

At day 4, VEGF expression levels are increased in RSV-infected lambs in both pretreatment groups. At day 6, VEGF expression levels are decreased in RSV-infected animals in both pretreatment groups (* $P < .05$; *** $P < .001$).

FIGURE 9.

Photomicrograph of immunohistochemical staining for Ki-67 antigen staining to assess cellular proliferation. Immunoreactive cells are present multifocally within the bronchiolar epithelium and within the interstitium (**A**, **B**). Bar = 100 μ m.

FIGURE 10.

Quantification of Ki-67 immunoreactive cells within lung tissue from lambs in all groups. At day 4, RSV-infected lambs pretreated with VEGF had increased numbers of Ki-67-immunoreactive cells compared to VEGF control (**A**). At day 6, RSV lambs pretreated with PBS had increased number of KI-67-immunoreactive cells compared to PBS control (**B**). RSV-infected lambs pretreated with rhVEGF showed no significant increase in Ki-67 immunoreactivity (* $P < .05$; *** $P < .001$).

TABLE 1

Treatment Groups for Assessing VEGF Pretreatment in RSV Disease

	Pretreatment	Inoculation
<i>n</i> = 4 (days 2, 4); <i>n</i> = 6 (day 6)	PBS	Medium
<i>n</i> = 4 (days 2, 4); <i>n</i> = 6 (day 6)	PBS	RSV
<i>n</i> = 4 (days 2, 4); <i>n</i> = 6 (day 6)	VEGF	Medium
<i>n</i> = 4 (days 2, 4); <i>n</i> = 6 (day 6)	VEGF	RSV

TABLE 2

Primers and Probe Sequences for Ovine Gene Expression Assessed by Real-Time qPCR

RSV	Fwd:	5'-GCTCTTAGCAAAGTCAAGTTGAATGA
	Rev:	5'-TGCTCCGTTGGATGGTGTATT
	Probe:	5'-6FAM-ACACTCAACAAAGATCAACTTCTGTCATCCAGC-TAMRA
SP-A	Fwd:	5'-TGACCCCTTATGCTCCTCTGGAT
	Rev:	5'-GGGCTTCCAAGACAAACTTCCT
	Probe:	5'-6FAM-TGGCTTCTGGCTCGAGTGCG-TAMRA
SP-D	Fwd:	5'-ACGTTCTGCAGCTGAGAAT
	Rev:	5'-TCGGTCATGCTCAGGAAAGC
	Probe:	5'-6FAM-TTGA CTAGCTGGCCACAGCCAGAACA-TAMRA
SBD-1	Fwd:	5'-CCATAGGAATAAAGGCGTCTGTGT
	Rev:	5'-CGCGACAGGTGCCAATCT
	Probe:	5'-6FAM-CCGAGCAGGTGCCCTAGACACATGA-TAMRA
IL-8	Fwd:	5'-TTCCAAGCTGGCTGTTGCT
	Rev:	5'-TTGACAGAACTGCAGCTTCACA
	Probe:	5'-6FAM-CCGCTTTCCTGCTCTCTGCAGCTC-TAMRA
IL-6	Fwd:	5'-GCTGCTCCTGGTGATGACTTC
	Rev:	5'-GGTGGTGTCATTTTGAATCTTCT
	Probe:	5'-6FAM-CTTTCCTACCCCGGTCCCTG-MGBNFQ
TNF α	Fwd:	5'-CAACCTGGGACACCCAGAAT
	Rev:	5'-TCTCAAGGAACGTTGCGAAGT
	Probe:	5'-6FAM-CAAGGGCCAGGTTCTTACCGGAA-TAMRA
IFN β	Fwd:	5'-TGGTTCTCCTGCTGTGTTCTC
	Rev:	5'-CGTTGTTGGAATCGAAGCAA
	Probe:	5'-6FAM-ACCACAGCTCTTCCAGGAGCTACA-TAMRA
VEGF	Fwd:	5'-GCCCACTGAGGAGTTCAACATC
	Rev:	5'-TGGCTTTGGTGAGGTTTGATC
	Probe:	5'-6FAMCCATGCAGATTATG-MGBNFQ
VEGF Receptor-1	Fwd:	5'-TCAGGTGAGCTTGGACAAAATG
	Rev:	5'-CACCGTGCAAGACAGCTTCA
	Probe:	5'-6FAM-CTGCGGAAGGAGAG-MGBNFQ
VEGF Receptor-2	Fwd:	5'-CCAGTGGGCTGATGACCAA
	Rev:	5'-GCAACAAACGGCTTTTCATGT
	Probe:	5'-6FAM-AAGAACAGCACGTTCTCGTCCGGG-TAMRA
Ubiquitin (ovine)	Fwd:	5'-GGTGGCTGTTAATTCTTCAG
	Rev:	5'-AAATGGCTAGAGTGCAGAACGAT
	Probe:	5'-6FAM-TTCATAATGCTCAGTGATGG-MGBNFQ
OvRPS15	Fwd:	5'-CGAGATGGTGGGCAGCAT
	Rev:	5'-GCTTGATTTCCACCTGGTTGA
	Probe:	5'-VIC-CCGGCGTCTACAACGGCAAGACC-TAMRA

Note. 6FAM or VIC = 5' fluorescent reporter dye; TAMRA = fluorescent quencher dye; MGBNFQ = minor groove binding nonfluorescent quencher.