Vascular changes after intra-amniotic endotoxin in preterm lamb lungs

Suhas G. Kallapur, Cindy J. Bachurski, Timothy D. Le Cras, Shubhada N. Joshi, Machiko Ikegami, and Alan H. Jobe

Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio 45229-3039

Submitted 6 August 2004; accepted in final form 18 August 2004

Kallapur, Suhas G., Cindy J. Bachurski, Timothy D. Le Cras, Shubhada N. Joshi, Machiko Ikegami, and Alan H. Jobe. Vascular changes after intra-amniotic endotoxin in preterm lamb lungs. Am J Physiol Lung Cell Mol Physiol 287: L1178–L1185, 2004. First published August 20, 2004; doi:10.1152/ajplung.00049.2004.—Chorioamnionitis is associated with preterm delivery and bronchopulmonary dysplasia (BPD), characterized by impaired alveolar and pulmonary vascular development and vascular dysfunction. To study the vascular effects in a model of chorioamnionitis, preterm lambs were exposed to 20 mg of intra-amniotic endotoxin or saline for 1, 2, 4, or 7 days and delivered at 122 days gestational age (term = 150 days). This intra-amniotic endotoxin dose was previously shown to induce lung maturation. The effect of intra-amniotic endotoxin on expression of endothelial proteins was evaluated. Mass spectrometry and supplemental oxygen for mechanical ventilation, pulmonary edema is observed (8). Similarly, in the preterm baboon mechanical ventilation-induced BPD model, decreased angiogenic growth factors and endothelial proteins and impaired microvascular development are present (34). Pulmonary hypertension is a frequent complication in infants with BPD and can cause significant morbidity and mortality (2, 17, 18). These studies suggest that decreased angiogenic growth factors and reduced endothelial proteins, vascular dysfunction, and remodeling are a central feature of BPD induced by postnatal insults. It is not known whether antenatal inflammation alone without exposure to mechanical ventilation causes pulmonary vascular changes and remodeling.

Vascular endothelial proteins VEGF-R2 and eNOS play an important role in pulmonary vascular development. Inhibition of the vascular endothelial growth factor-receptor 2 (VEGF-R2) signaling (19, 29) or lack of heparin-binding VEGF isoforms VEGF-165 and VEGF-188 in the developing lung of rodents causes decreased pulmonary vascular development and alveolarization (36). Deficiency of eNOS also causes impairment of alveolarization and reduced pulmonary vascular development with exposure to mild hypoxia (6). Decreased alveolar growth, reduced eNOS expression and signaling, as well as abnormal pulmonary vascular development also occur in preterm sheep and baboons exposed to mechanical ventilation and supplemental oxygen for >2 wk (3, 8, 9, 12). Expression of these endothelial proteins after antenatal inflammation is not known.

We have developed fetal preterm lamb models in which intra-amniotic endotoxin injection given as either a single dose or as a continuous infusion causes impaired alveolarization in preterm lambs (35, 50). In these fetal models, reduced alveolarization similar to BPD occurs without exposure to postnatal insults, such as oxygen or mechanical ventilation. Vascular changes have not been studied in this model.

We recently showed that the angiostatic chemokine IP-10 mRNA is induced in the fetal lamb lung 1–4 days after intra-amniotic endotoxin exposure (24). We therefore hypothesized that intra-amniotic endotoxin would inhibit expression of proteins critical for endothelial function followed by vascular remodeling in the preterm lung. We evaluated the time course of expression of endothelial proteins and studied remodeling in the muscular and adventitial compartments in the preterm lung exposed to antenatal endotoxin.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
PECAM-1 cDNA was excised and the plasmid relegated. The result-
P32]UTP-labeled probes for 16 h at 55°C. Single-stranded RNA
(protected fragment 355 bp). T7 RNA polymerase was used to generate an antisense riboprobe
(fragment 198 bp). The plasmid pGEMT-sTie1 was cut with
polymerase was used to generate an antisense riboprobe (protected
fragment 198 bp). The plasmid pGEMT-VEGF 165–188 was linearized with
BamHI/H18528 and SP6 RNA
NcoI, and SP6 RNA
BsaI. The
was digested with RNase A/T1 (Pharmingen, San Diego, CA). RNase
was then inactivated, and protected RNA was precipitated using the
was demarcate the muscularis media. Ten arterioles per lamb with measurements of
50 μm external diameter accompanying the terminal bronchioles (identified by morphologic criteria) were measured by a blinded observer, and three to four lambs were evaluated per group. Only transversely sectioned airways were evaluated to minimize distortion of arteriolar muscularis media. Morphometric measurements included thickness of the muscularis media, external vessel diameter, external vessel area, and area of smooth muscle (vessel lumen external area-internal area) and were analyzed using Meta-
morph version 6.1 software (Universal Imaging, Downingtown, PA) on digitally acquired images. The wall thickness was expressed as
[(2 × medial wall thickness/external diameter)] × 100.

The adventitial fibrosis was evaluated by Masson’s trichrome
staining. Ten arterioles per lamb with measurements of <50 μm external diameter accompanying the terminal bronchioles were scored by a blinded observer for collagen staining, and three to four lambs were evaluated per group. A qualitative scoring system was used to evaluate adventitial fibrosis: score 0 = none, score 1 = mild, score 2 = moderate, and score 3 = severe.

Statistics. The RNA, protein quantitation, and vessel morphometric and scoring results were expressed as means ± SE. Comparisons between endotoxin-exposed animals and controls were made with two-tailed unpaired t-tests, two-tailed Mann-Whitney nonparametric tests, or two-way Kruskal-Wallis nonparametric ANOVA, as appropriate. Significance was accepted at P < 0.05.
RESULTS

Cord blood hematocrit and blood gas. The cord blood hematocrit, PO2, PCO2, and pH were similar between control and endotoxin-exposed preterm lambs. There were no differences in the cord blood hematocrit, PO2, PCO2, and pH values within the different endotoxin-exposed subgroups. The cord blood PO2 level was 13.8 ± 0.9 mmHg in controls and 14.4 ± 0.7 mmHg in endotoxin-exposed animals. The cord blood hematocrit was 38 ± 1 in controls and 34.5 ± 0.6 in endotoxin-exposed animals. There were no fetal deaths in this study, either in the control or the endotoxin-exposed groups.

Intra-amniotic endotoxin increases BALF protein. Protein concentration was measured in the BALF (Fig. 1). BALF recovery volumes and the body weights were similar in control and endotoxin-exposed preterm lambs (267 ± 17 ml and 2.4 ± 0.1 kg vs. 258 ± 12 ml and 2.3 ± 0.1 kg, respectively, not statistically different). BALF protein content increased by 2.3-fold 2 days after intra-amniotic endotoxin exposure and returned to control levels at 4 days.

Intra-amniotic endotoxin decreases eNOS protein expression. eNOS plays a critical role in regulating pulmonary vascular tone and modulating pulmonary vascular development (6, 13). Western blot analysis showed that eNOS protein expression in lung homogenates decreased 2–4 days after intra-amniotic endotoxin with a maximum decrease to 40% of the control value at 4 days (Fig. 2A). At 7 days after intra-amniotic endotoxin exposure, lung eNOS protein levels approached control levels. The cellular localization of eNOS protein expression was evaluated by immunohistology. In the control preterm lambs, eNOS immunostaining was detected in lung vascular endothelial cells (Fig. 2B). Compared with controls, eNOS staining was faint in the endothelial cells of the small pulmonary vessels at 1, 4, and 7 days after intra-amniotic endotoxin exposure (Fig. 2, C–E). Interestingly, eNOS staining in the endothelium of the large vessels was similar between the controls and the endotoxin-exposed lambs (Fig. 2, B–E, Insets).

Intra-amniotic endotoxin downregulates VEGF expression. VEGF signaling is known to regulate the activity of eNOS (51). We therefore evaluated the expression of VEGF mRNA in the lung by RNase protection assay (RPA; Fig. 3A). The antisense VEGF RPA probe was designed to differentially detect two splice forms (VEGF-165 and VEGF-188). VEGF-165 mRNA expression decreased 2 and 4 days after intra-amniotic endotoxin exposure to 55% of the control levels (Fig. 3B). The expression of VEGF-188 mRNA paralleled the decrease in VEGF-165 mRNA expression. VEGF protein levels were evaluated by Western blot analysis using two different antibodies (Fig. 3C). In preliminary experiments using sheep lung homogenates, we found that the rabbit polyclonal antibody (sc-152) detected the dimeric VEGF (42 kDa) but did not detect the VEGF monomer. The mouse monoclonal antibody (sc-7269) detected only the monomeric VEGF (21 kDa). However, a high concentration of 4.4% B-mercaptoethanol only incompletely reduced the sheep lung VEGF disulfide bonds. To eliminate variability resulting from differential reductions between the groups and to accurately define the VEGF species, VEGF immunoblot densitometry was performed initially with the monoclonal anti-VEGF antibody followed by the polyclonal anti-VEGF antibody using recombinant mouse and human VEGF standards. Consistent with the decreased VEGF-165 mRNA 2 and 4 days after intra-amniotic endotoxin exposure, both the dimeric and the monomeric VEGF protein were decreased 4 and 7 days after intra-amniotic endotoxin exposure (Fig. 3D). VEGF protein levels were maximally decreased to 40% of control levels 7 days after intra-amniotic endotoxin.

Intra-amniotic endotoxin decreases endothelial protein expression. PECAM-1 (CD31), Tie-2, and VEGF-R2 are important for endothelial cell development and function (41, 42). We therefore evaluated the expression of these proteins in the preterm lung after intra-amniotic endotoxin exposure (Fig. 4A). PECAM-1 protein levels decreased to 50% of the control levels at 2 and 4 days after intra-amniotic endotoxin (Fig. 4B). The Tie-2 protein levels decreased to 45% of control levels at 2 days and 75% of control levels at 4 days after intra-amniotic endotoxin. VEGF-R2 protein decreased to 35–40% of the control values at 1–4 days after intra-amniotic endotoxin. PECAM, Tie-2, and VEGF-R2 protein levels approached control values by 7 days after intra-amniotic endotoxin. Consistent with the decreased protein expression, PECAM mRNA and Tie-1 mRNA also decreased to 60 and 52% of the control value, respectively (data not shown).

Intra-amniotic endotoxin causes arteriolar smooth muscle hypertrophy and increases in the vascular adventitia. To determine whether intra-amniotic endotoxin increased muscularization of small pulmonary arteries, the medial wall thickness was measured by morphometry. Resistance arterioles (<50 μm external diameter) accompanying terminal bronchioles of preterm lambs were identified using α-smooth muscle actin immunostaining. The median arteriolar external diameter was 25 μm (range 15–45 μm) in both controls and endotoxin-exposed lambs. Arteriolar smooth muscle thickness increased from 39% in controls to 47% 4 days after intra-amniotic endotoxin and to 53% 7 days after intra-amniotic endotoxin (Fig. 5A, P < 0.05). Consistent with increased smooth muscle wall thickness, the area occupied by smooth muscle/total arteriolar area also increased from 0.62 in controls to 0.74 4 days after intra-amniotic endotoxin and 0.77 7 days after intra-amniotic endotoxin (Fig. 5B, P < 0.05). In addition to the increased arteriolar smooth muscle thickness after intra-amniotic endotoxin, increased adventitial layer fibrosis and cellularity was seen in sections of preterm lamb lungs 7 days after intra-amniotic endotoxin compared with controls (Fig. 5, C–E). To further characterize changes in the vascular adventitia,
Masson’s trichrome staining for detection of collagen deposition was performed (Fig. 5, F-H). The adventitial fibrosis (blue staining) score for control lambs was 0.8 ± 0.1. The adventitial fibrosis score increased 4 days after intra-amniotic endotoxin to 1.63 ± 0.16 and 7 days after intra-amniotic endotoxin to 2.1 ± 0.14 (P < 0.01 for both endotoxin-exposed groups vs. controls).

**DISCUSSION**

These results are the first to demonstrate the effects of antenatal inflammation alone without mechanical ventilation or oxygen exposure on the fetal pulmonary vasculature. Expression of VEGF, eNOS, PECAM-1, Tie-2, and VEGF-R2 was reduced for up to 7 days after intra-amniotic endotoxin. Vascular remodeling, as demonstrated by smooth muscle hypertrophy and increased adventitial collagen deposition in resistance arterioles, occurred 4 and 7 days after intra-amniotic endotoxin exposure.

The effects of intra-amniotic endotoxin are different from systemic (intramuscular or intravenous) endotoxin exposure. Compared with the 20-mg intra-amniotic endotoxin dose used in this study, systemic injection of far lower doses (〜0.05 mg) increases lung vascular permeability in adult sheep and causes death in preterm lambs (10, 23). This animal model differs from infants exposed to chorioamnionitis in the lack of exposure to live replicating bacteria. We have previously reported minimal systemic inflammation and no placental inflammation after 20 mg of intra-amniotic endotoxin (25). In this study, no fetal acidosis or fetal deaths were observed after intra-amniotic endotoxin. In previous studies, a lower intra-amniotic endotoxin dose of 0.1 or 1 mg inconsistently induced lung inflammation and surfactant synthesis (22, 27). Larger doses of 100 mg of intra-amniotic endotoxin caused comparable lung maturation responses without inducing fetal death, decreasing fetal growth, or causing miscarriage in the ewe (22). The mixing of the endotoxin with a relatively large volume (〜0.5
liters) of amniotic fluid upon injection and the likely differences between the respiratory epithelial vs. endothelial responsiveness to endotoxin probably contribute to the differences between the intra-amniotic vs. systemic endotoxin exposure. These results suggest that the vascular effects observed in this study occurred as a result of the endotoxin-induced fetal lung inflammation and not because of direct endotoxin effects on the vascular endothelium.

In the preterm lamb model used in this study, maximum influx of activated inflammatory cells and induction of proinflammatory cytokines and the angiostatic interferon inducible chemokine (interferon inducible protein-10 and monokine induced by gamma interferon) mRNAs in the lung occurs 1–2 days after intra-amniotic endotoxin (24, 25, 27). Airway surfactant proteins and lipids were increased 7 days after intra-amniotic endotoxin (25). Among the endothelial proteins, striking reductions in the expression of eNOS and VEGF-R2 were observed in this study as early as 1 day after endotoxin exposure. We previously reported no significant change in numbers of alveolar type II cells after intra-amniotic endotoxin in this model (26). One possible explanation for reduced endothelial proteins is reduced epithelial and vascular surface area after endotoxin exposure. We have previously reported decreased alveolarization at 7 days after intra-amniotic endotoxin exposure (50). Taken together, these data are consistent with the hypothesis that intra-amniotic endotoxin-induced inflammatory changes and/or the induction of angiostatic chemokines may decrease expression of proteins important for endothelial cell function. The vascular changes along with the fetal lung inflammation may contribute to decreased alveolarization.

In the present study, eNOS protein content in the lung was reduced to ~50% of the control levels 1–4 days after exposure.
to intra-amniotic endotoxin. By immunostaining, eNOS expression appeared to be selectively reduced in the small pulmonary arteries compared with large conducting vessels. Reductions in lung eNOS protein content and decreased responsiveness to inhaled nitric oxide have been reported after 3 wk of chronic mechanical ventilation in preterm lambs (9, 33). Mice homozygous for the targeted deletion of eNOS have decreased postnatal survival, impaired alveolarization, and severe pulmonary hypertension after exposure to mild hypoxia, suggesting a critical requirement of eNOS for postnatal survival (6, 13, 14, 37). Taken together, these studies along with the results of the present study suggest that eNOS deficiency may contribute to reduced alveolarization and remodeling changes after exposure to intra-amniotic endotoxin.

VEGF signaling is essential for endothelial cell proliferation, survival, and angiogenesis (15). A downstream target of VEGF signaling is nitric oxide and nitric oxide synthase expression and activity (51). Inhibition of VEGF or the VEGF receptor signaling causes reduced alveolarization, decreased pulmonary vascular development, and pulmonary hypertension.
in the developing lung (16, 19, 29). VEGF mRNA expression in the fetal lambs at the gestation used in this study is largely localized to type II pneumocytes (data not shown). After intra-amniotic endotoxin, expression of surfactant proteins B and C, also products of type II pneumocytes, is markedly induced (4) at the same time that VEGF mRNA and protein are decreased. This result suggests that the effects of antenatal inflammation on VEGF expression are the result of specific gene regulation. In the present study, although VEGF mRNA levels did not decrease for 2 days, the VEGF-R2 expression decreased 1 day after intra-amniotic endotoxin. Consistent with the published literature, our data suggest that decreased VEGF signaling (VEGF-R2 expression) may contribute to reduced eNOS expression.

Preterm baboons exposed to mechanical ventilation and oxygen have decreased lung PECAM-1, VEGF, and VEGF-R1 (Flt-1) expression (34). In the present study, decreased PECAM-1, Tie-2, eNOS, and VEGF-R2 expression was observed. PECAM-1 plays a critical role in the trans-endothelial migration of inflammatory cells and angiogenesis (43). Angiopoietin-1 and angiopoietin-2 signal via Tie-2, a protein tyrosine kinase receptor that plays an essential role in angiogenesis and vessel remodeling (31, 41). The present study and published literature suggest that a combined decrease in VEGF and angiopoietin signaling after antenatal inflammation would likely have adverse consequences on vascular function and development. Although the quantitation of viable endothelial cells or apoptosis was not done, recovery of expression of several endothelial proteins suggests a functional impairment of endothelial cells rather than cell death.

Pulmonary arteriolar smooth muscle hypertrophy contributes to pulmonary hypertension and occurs in response to chronic hypoxia, increased shear stress, and endothelial cell injury (1, 38, 39). In this study, hematocrits and umbilical arterial oxygen levels were similar between controls and endotoxin-exposed animals, suggesting lack of significant hypoxemia in endotoxin-exposed fetuses. Similarly, the fetal pulmonary circulation is exposed to low shear stress resulting from low blood flow. Therefore, increased shear stress is an unlikely explanation for smooth muscle hypertrophy. In transgenic mice, deficiency of eNOS increases susceptibility of mice to hypoxia-induced pulmonary arteriolar smooth muscle hypertrophy (13). Decreased VEGF-R2 signaling and eNOS expression in this study may have contributed to arteriolar smooth muscle hypertrophy. The increase in arteriolar smooth muscle thickness in 7 days intra-amniotic endotoxin-exposed animals relative to controls in this study is similar in magnitude to the smooth muscle hypertrophy reported in preterm lambs after intrauterine patent ductus arteriosus ligation (5). In our study, increases in the adventitial cellularity and collagen deposition were seen in the small pulmonary arteries 4 and 7 days after intra-amniotic endotoxin exposure, coinciding with medial hypertrophy. Although the adventitial cell numbers were not quantified, there appeared to be increased numbers of cells in the adventitia. These adventitial cells were not positive for α-smooth muscle actin staining, suggesting that they were fibroblasts and not myofibroblasts. Other injurious stimuli, such as hypoxia, ischemia and reperfusion, tissue damage, overstretch injury, inflammation, and reactive oxygen species, are known to activate and induce proliferation of adventitial fibroblasts (40, 44). Increased adventitial collagen deposition was reported in infants with BPD (45). Our results demonstrate that exposure to antenatal inflammation causes remodeling changes both in the medial and adventitial layers of small pulmonary arteries, which should increase pulmonary vascular resistance.

In summary, the present study demonstrates reduced endothelial protein expression and vascular remodeling in the preterm lamb lung after 20 mg of intra-amniotic endotoxin.

REFERENCES


29. Le Cras TD, Markham NE, Tuder RM, Voelkel NF, and Abman SH. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmo-


32. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. Protein mea-

33. MacRitchie AN, Albertine KH, Sun J, Lei JS, Jensen SC, Freestone AA, Clarr PM, Dahl MJ, Godfrey EA, Carlton DP, and Bland RD. Reduced endothelial nitric oxide synthase in lungs of chronically venti-


45. Stocker JT. Pathologic features of long-standing “healed” bronchopulmo-


