Excess soluble vascular endothelial growth factor receptor-1 in amniotic fluid impairs lung growth in rats: linking preeclampsia with bronchopulmonary dysplasia

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BRONCHOPULMONARY DYSPLASIA (BPD) is the chronic lung disease of infancy that follows premature birth and is characterized by impaired lung growth due to early lung injury. BPD is a common and severe complication of preterm birth (19, 59, 66). Infants with BPD have prolonged respiratory insufficiency and are at high risk for pulmonary hypertension (39, 65), lung infections (51, 78), recurrent hospitalizations (63), exercise intolerance (30, 74, 75), and adverse neurodevelopmental outcome (3). Past studies have identified significant contributions to the pathogenesis of BPD from prematurity, side effects of respiratory support (9, 18, 52), and complications of neonatal illness (5, 27, 44). Although the use of antenatal steroids, surfactant, and improvements in ventilator and oxygen therapies have markedly altered the clinical course of preterm infants, the incidence of BPD has not decreased in the very premature infants during the past decade (59, 66). Late respiratory complications of premature birth and BPD generally reflect the effects of persistent abnormalities in distal lung structure, which include decreased alveolarization and a dysmorphic pulmonary vascular bed (14).

Recently, the impact of an adverse intrauterine environment on impairing neonatal lung growth and worsening respiratory outcomes of preterm infants has been recognized by both clinical (8, 10, 28, 31, 41, 54, 55) and experimental studies (29, 47, 69, 71). Among diverse antenatal factors, preeclampsia (PE) is shown to be independently associated with a high risk for BPD, but the underlying mechanism is unknown (28, 31, 40, 79).

PE is the most common complication of pregnancy and is due to maternal endothelial dysfunction that leads to hypertension, proteinuria, and other complications (61, 62). Moreover, maternal PE is a frequent cause of preterm birth (7), accounting for nearly 20% of delivery before 28 wk gestational age (49), and significantly increases perinatal and neonatal morbidities (61, 62). Placental overproduction of soluble vascular endothelial growth factor (VEGF) receptor-1 (soluble fms-like tyrosine kinase-1, also known as sFlt-1), which inhibits VEGF signaling mainly through trapping free VEGF (38, 60), causes maternal endothelial dysfunction and plays a central role in the pathogenesis of PE (37, 43, 46, 48). The magnitude of excess sFlt-1 in maternal serum not only correlates with the severity of PE (48, 73), but serum levels of sFlt-1 in the mother are also inversely related to gestational age and birth weight of the newborn (73). In addition to high maternal serum levels, sFlt-1 is markedly increased in amniotic fluid during the second and third trimesters of pregnancies complicated by PE (64, 76, 77). Whether exposure of the fetal lung to excess intra-amniotic sFlt-1 can impair lung VEGF signaling and contribute to the pathogenesis of BPD is unknown. VEGF, a key regulator of angiogenesis (12, 56), plays a critical role in directing vascular and alveolar growth in the developing lung. Previous studies have shown that VEGF inhibition during the early neonatal period results in persistent abnormalities of alveolar and pulmonary vascular structures into and beyond infancy, which are characteristic of pathological changes in human BPD (32, 42, 67). However, whether transient impairment of VEGF signaling in the fetus is sufficient to cause sustained disruptive postnatal lung growth has not been studied.


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To determine mechanisms that may link PE with a high risk for BPD, we hypothesized that antenatal exposure to excess sFlt-1 disrupts normal lung development through impaired VEGF signaling in utero. We further hypothesized that fetal exposure to elevated levels of intra-amniotic sFlt-1 is sufficient to cause sustained abnormalities of lung structure into infancy. We report that intra-amniotic sFlt-1 treatment during late gestation impaired lung VEGF signaling and increased apoptosis of lung cells in newborn rats, which were followed by reductions in alveolarization and lung vascular growth along with biventricular hypertrophy during infancy. These findings demonstrate biological plausibility underlying epidemiological evidence that links PE with BPD.

MATERIALS AND METHODS

Animals

All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver’s altitude (1,600 meters; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) for at least 1 wk before giving birth. Animals were fed ad libitum and exposed to day-night cycles alternatively every 12 h. Rats were killed with an intraperitoneal injection of pentobarbital sodium (0.3 mg/g body wt; Fort Dodge Animal Health, Fort Dodge, IA).

Study Design

Intra-amniotic sFlt-1 administration. At 20 days gestation (term: 22 days), pregnant rats were prepared for receiving intra-amniotic injections. The timing of injection during the late canalicular stage of lung development in the rat was selected to parallel a similar stage of human lung development in 24- to 26-wk premature newborns who are at the highest risk for BPD. After premedication with buprenorphine (0.01–0.05 mg/kg, intramuscular injection), laparotomy was performed on pregnant rats under general anesthesia with 1–2% isoflurane inhalation via a face mask (Anesthesia machine: Matrix by Midmark, model VIP3000). During anesthesia and laparotomy, pregnant rats were kept on a heating pad for preventing hypothermia. Rats with isoflurane inhalation, as described above. The fetus in the amniotic sac closest to the right ovary was first delivered, which was followed by delivery of the rest of the fetuses in a counterclockwise sequence, to identify fetuses exposed to intra-amniotic injections. The total number of amniotic sacs in each mother rat was further verified at the time of delivery. The main reason for performing cesarean section instead of allowing vaginal delivery is to identify the fetuses exposed to intra-amniotic injections, based on the order of the amniotic sacs and their anatomic locations related to the ovaries. All of the rat pups in the injected amniotic sacs were delivered within 5 min after the onset of anesthesia. Maternal rats were then killed with pentobarbital sodium. Newborn rats were immediately placed on a heating pad to avoid hypothermia and were dried manually with gauze sponges. Pups received no supplemental oxygen or artificial ventilation at birth. The survival rate at birth was recorded. Within 30 min after birth, the pups were weighed and placed with foster mother rats in regular cages. For the first 24 h of life, the newborn pups were monitored closely for mortality or signs of respiratory distress.

Rat lungs were harvested at birth for Western blot analysis and at birth and 14 days of age for histological assessment. Hearts were dissected and weighed at birth and at 7 and 14 days of age. Three to nine rats were studied in each group for each measurement at each time point. Survival of the infant rats was monitored and recorded daily from birth throughout the study period. Survival rate was calculated as the number of survived pups divided by the number of sacs that received intra-amniotic injection in each given litter. Body weight was measured at birth and at the time of being killed for study measurements.

Study Measurements

Tissue for histological analysis. Animals were killed with intraperitoneal pentobarbital sodium. A catheter was placed in the trachea, and the lungs were inflated with 4% paraformaldehyde and maintained at 20 cmH2O pressure for 60 min. A ligature was tightened around the trachea to maintain pressure, and then the tracheal cannula was removed. Lungs were then immersed in 4% paraformaldehyde at room temperature for 24 h for fixation. A 2-mm-thick transverse section was taken from the midplane of the right lower lobe and left lobe of the fixed lungs per animal, respectively, to process and embed in paraffin wax.

Immunohistochemistry. Slides with 5-μm paraffin sections were stained with hematoxylin and eosin for assessing alveolar structures and with von Willebrand Factor (vWF), an endothelial cell-specific marker, for quantifying vessel density.

Morphometric analysis. RAC, mean linear intercept (MLI), and other indexes of alveolar structure and pulmonary vessel density were determined by standard morphometric techniques, as outlined below. In each animal, at least 5 measurements were obtained for RAC, at least 10 images were processed for computer-assisted image analysis of alveolar structure, and at least 10 high-power fields were examined for measuring pulmonary vessel density.

RADIAL ALVEOLAR COUNTS. Alveolarization was assessed by the RAC method of Emery and Mithal as described (16, 17). Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall. From the center of the respiratory bronchiole, a perpendicular line was dropped to the edge of the acinus connective
tissues or septum or pleura, and the number of septae intersected by this line was counted.

MLI AND OTHER INDEXES OF ALVEOLAR STRUCTURE. Measurements of MLI, nodal point density, and interstitial thickness were performed with a computer-assisted image analysis program. Briefly, the images were captured on a Zeiss Axioskope2 microscope, using the ×10 objective and captured as a high-resolution TIFF image by a MicroPublisher digital camera (2,560 × 1,940 pixel resolution; Qimaging, Burnaby, Canada). The fields with large airways or vessels were avoided. These images were processed as previously described (4).

PULMONARY VESSEL DENSITY. Pulmonary vessel density was determined by counting vWF-stained vessels with an external diameter at 50 μm or less per high-power field. The fields containing large airways or vessels with external diameter >50 μm were avoided.

Indexes of Right Ventricular Hypertrophy and Left Ventricular Hypertrophy

The right ventricle (RV) and left ventricle plus septum (LV+S) were dissected and weighed. The ratios of RV to LV+S weights (RV/LV+S%) and RV/body weights (RV/BW%) were determined to evaluate right ventricular hypertrophy (RVH). The ratio of LV+S to body weight (LV+S/BW%) was determined to evaluate left ventricular hypertrophy (LVH).

Western blot analysis. Frozen lung samples were homogenized in ice-cold radioimmunoprecipitation buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mg/ml phenylmethylsulfonyl fluoride) with protease inhibitor (Pierce, Rockford, IL) and phosphatase inhibitor cocktail (Calbiochem, Gibbstown, NJ). Samples were centrifuged at 1,500 g for 20 min at 4°C to remove cellular debris. Protein content in the supernatant was determined by the Bradford method (11), using BSA as the standard. Next, 25 μg [for total VEGF, endothelial nitric oxide synthase (eNOS), and active caspase-3] or 50 μg [for total and phosphorylated vascular endothelial growth factor receptor 2 (VEGFR2)] of protein sample per lane was resolved by SDS-PAGE, and proteins from the gel were transferred to polyvinylidene difluoride membrane. Blots were blocked for 1 h or overnight in 5% nonfat dry milk in PBS with 0.1% Tween 20. For total VEGF and eNOS, the blots were incubated for 1 h at room temperature with rabbit anti-human polyclonal VEGF antibody (sc-...
was similar between the two groups from birth through day 14. The rate of body weight gain was not different between the two groups at each time point evaluated, statistically not different from saline control, and no mortality was found between the two groups during the 14-day study period.

Survival Rate and Body Weight

A total of three litters of fetal rats received intra-amniotic saline injections, and four litters received intra-amniotic sFlt-1 treatment. Both control and sFlt-1 groups had 100% survival at birth (Fig. 1A). In both groups, newborn rats appeared pink and vigorous without evidence of cyanosis or respiratory distress, as closely monitored for the first 24 h of life. In the two litters kept through day 14 in each group, the survival rate of sFlt-1 rats decreased to 80% at day 1, statistically not different from the 95% of survival rate in saline control. No mortality was found at and beyond day 2 in either group. There was no difference in body weights between sFlt-1 and saline rats at birth, and days 7 and 14 (Fig. 1B). The rate of body weight gain was similar between the two groups from birth through day 14.

Lung Histology and Morphometric Analyses

Figure 2 shows the effects of intra-amniotic sFlt-1 treatment on alveolarization in 14-day-old rats. The distal airspace appeared simplified with decreased septation and alveoli in sFlt-1 rats as compared with saline control (Fig. 2A). By morphometric analysis, sFlt-1 rats had decreased RAC (P < 0.0001), increased MLI (P < 0.0001), and decreased nodal point density (P < 0.0001) compared with saline control; interstitial thickness was not different between the two groups (Fig. 2B).

The effects of intra-amniotic sFlt-1 treatment on pulmonary vascular growth in 14-day-old rats are shown in Fig. 3. Pulmonary vessel density was decreased by 38% in sFlt-1 rats compared with saline control (P < 0.0001). There was no difference found between the male and female in the sFlt-1 group regarding lung histology and morphometric analyses.
RVH and LVH Indexes

The effects of intra-amniotic sFlt-1 treatment on heart weights in infant rats are shown in Fig. 4. The RV/LV+S and RV/BW ratios (Fig. 4, A and B), both of which are proportional to the degree of RVH, were comparable between sFlt-1 rats and saline control at birth and at day 7, respectively. Both parameters in sFlt-1 rats increased above control values at day 14 (P < 0.05). Similarly, the LV+S/BW ratio (Fig. 4C), an index of LVH, was similar between sFlt-1 and saline groups at birth and at day 7, respectively, but then increased in sFlt-1 rats at day 14 to 19% above control values (p < 0.05). From birth through day 7, the RVH and LVH indexes decreased in both groups (Fig. 4, A–C). From day 7 to day 14, the RV/BW ratio tended to further decrease in the control, whereas it trended up in sFlt-1 rats (Fig. 4B). During this period, the LV+S/BW ratio nearly further decreased in the control, but it remained unchanged in sFlt-1 rats (Fig. 4C). The time course suggests neonatal onsets of progressive RVH and LVH in sFlt-1 rats. At day 14, the effect of intra-amniotic sFlt-1 treatment on increasing RV/BW% was more striking in female infant rats than male (Fig. 4D). There was no apparent gender-related difference on LV+S/BW% in sFlt-1 rats (Fig. 4E); n = 4–8 animals/group.

Effects of Intra-Amniotic sFlt-1 on VEGF Signaling in the Newborn Rat Lung

To assess the effects of intra-amniotic sFlt-1 treatment on lung VEGF signaling at birth, phosphorylated VEGFR2, total VEGFR2, total VEGF, and eNOS proteins were measured by Western blot analyses on newborn rat lung homogenates. Lung phosphorylated VEGFR2 protein was decreased in sFlt-1 rats compared with saline controls, sFlt-1 rats showed decreased ph-VEGFR2 (A), unchanged total VEGF2 (B), and a decreased ratio of ph-VEGFR2 to total VEGF2 proteins (C) in the lung at birth; n = 3–5 animals/group. NS, not significant.
compared with saline control ($P < 0.05$; Fig. 5A), whereas lung total VEGFR2 protein was comparable between the two groups (Fig. 5B). The ratio of phosphorylated VEGFR2 to total VEGFR2 proteins in the sFlt-1 group was 46% below control values in the newborn rat lungs ($P < 0.01$; Fig. 5C). Total lung VEGF (Fig. 6) and eNOS protein contents (Fig. 7) did not differ between sFlt-1 and control groups at birth.

**Effects of Intra-Amniotic sFlt-1 on Apoptosis in the Newborn Rat Lung**

To evaluate the effects of intra-amniotic sFlt-1 treatment on activity of apoptosis in newborn rat lungs, active caspase-3 protein was analyzed by Western blot on lung homogenates, and showed elevation by 76% in sFlt-1 rats ($P < 0.01$; Fig. 8A). Consistent with Western blot analysis, active caspase-3 signals remarkably increased in sFlt-1 rats as compared with saline control, in immunofluorescence staining on newborn rat lung sections (Fig. 8B).

To identify which cells express active caspase-3 in the newborn lung, immunofluorescent staining was performed with active caspase-3 and one of the markers of variable lung cell types on newborn rat lung sections. Compared with scarce signals of active caspase-3 on vWF-positive cells in saline control (Fig. 9A), sFlt-1 rats demonstrated colocalization of vWF and active caspase-3 in microvessels (Fig. 9B) but not in larger pulmonary vessels (Fig. 9C). To further identify nonendothelial cells stained with active caspase-3 in the distal airspace of sFlt-1 rats (Fig. 9B), immunofluorescence staining was performed for active caspase-3 with vimentin, α-SMA, and SP-C precursor, respectively. Vimentin is expressed in mesenchymal and endothelial cells, α-SMA is a marker of myofibroblasts in distal airspace, and SP-C precursor is produced exclusively by type-2 alveolar epithelial cells. Many cells that were positive for active caspase-3 coexpressed vimentin in the interstitium of distal airspace (Fig. 10A), indicating the localization of active caspase-3 in mesenchymal cells in addition to endothelial cells. There was no colocalization of active caspase-3 and α-SMA (Fig. 10B), indicating that those active caspas-3 (+) mesenchymal cells are not myofi-

**DISCUSSION**

We found that excess sFlt-1 in amniotic fluid during the late canalicular and early saccular stage of lung development reduces VEGF signaling and increases apoptosis in the newborn rat lung, which is followed by sustained reductions in alveolarization and pulmonary vascular growth during infancy. In addition, infant rats that are exposed to excess intra-amniotic sFlt-1 develop RVH and LVH over time. These findings suggest that the increased risk for BPD and vascular dysfunction in infants with maternal PE may result from fetal exposure to elevated levels of intra-amniotic sFlt-1.

For more than a decade, epidemiological studies have shown an increased risk of BPD in preterm infants born to preeclamptic mothers (28, 31, 40, 79), but the underlying mechanism was unknown. The present study shows that excess intra-amniotic sFlt-1 is sufficient to cause sustained abnormalities of lung structure during infancy. This effect is likely mediated through impairing lung VEGF signaling in the fetus, leading to increased endothelial and mesenchymal apoptosis in the distal airspace of the newborn. These findings further demonstrate the critical role of VEGF signaling in directing vascular and alveolar growth in the developing lung. Moreover, the present study reveals that intact VEGF signaling is required to maintain both endothelial and mesenchymal cell survival during lung development. Our finding confirms and extends past studies that endothelial survival relies on VEGF signaling through phosphorylation of VEGFR2 (20) and that inhibition of VEGFR2 injures pulmonary endothelium in the fetus (80) and neonates (68). A recent study shows that VEGF mediates endothelial-mesenchymal cross-talk to drive morphogenesis of testis (15). Future work is needed to determine whether VEGF signaling directly regulates mesenchymal cells or through endothelial cells in the developing lung.

This is the first study demonstrating that primary inhibition of VEGF in the fetus results in sustained abnormalities of lung growth.
structure during infancy. Previous studies have shown that intra-amniotic endotoxin or antenatal dexamethasone treatment downregulates lung VEGF or VEGFR-2 expression at birth and impairs alveolarization through late infancy in rats (57, 69). The present study highlights that antenatal impairment of lung VEGF signaling may link the sustained disruption of infant lung growth with preceding intrauterine stimuli, such as chorioamnionitis, antenatal dexamethasone, and PE, in animal models. Past studies have shown that neonatal VEGF inhibition reduces angiogenesis as well as alveolar growth in the developing lung, but the mechanisms are poorly understood (22, 42, 50, 67, 81). With impaired VEGF signaling in utero, as shown in the current study, mesenchymal cell apoptosis could further compromise vascular growth in the developing lung, given mesenchymal cells are capable of differentiating into perivascular cells to stabilize newly forming endothelial channels (2, 45). Future study is needed to better characterize the α-SMA-negative mesenchymal lung cells that undergo apoptosis under suppression of VEGFR2 signaling, in order to delineate whether mesenchymal injury due to VEGF inhibition is directly involved in the impaired formation of secondary septae in the developing lung.

This rat model of PE demonstrates that excess sFlt-1 in amniotic fluid impairs lung VEGF signaling in the fetus. Following intra-amniotic sFlt-1 treatment, the reduction in phosphorylated VEGFR2 in the presence of intact total VEGFR2 indicates a shortage of lung VEGF to activate VEGFR2 in the newborn lung. As in clinical PE, excess intra-amniotic sFlt-1 may directly reach the fetal lung via fetal breathing, or through an intramembranous pathway and fetal swallowing to enter the fetal circulation and then arrive in the fetal lung. Bidirectional flow of amniotic fluid across human fetal trachea has been proven by Doppler velocimetry during normal fetal breathing movements (36). Moreover, fetal pulmonary aspiration of intra-amniotic sFlt-1 is supported by the finding that muscle paralysis inhibits lung deposition of intra-amniotic iron dextran in fetal rabbits, whereas iron accumulates in control fetal lungs (21). In addition, substances in the amniotic cavity can be transferred into the fetal circulation through an intramembranous pathway (23–26), which is characterized by a micro-
scopic network of fetal vasculature on the fetal surface of the placenta (23, 24).

This study also reports the late development of RVH in infant rats treated with intra-amniotic sFlt-1, suggesting the progressive onset of pulmonary hypertension after exposure to the fetal environment of PE. Our findings are interesting not only because pulmonary hypertension can complicate BPD (39, 65) but also because offspring of mothers with PE are at risk for pulmonary vascular dysfunction later in life (33).

Similarly, we found LVH in infant rats exposed to excess sFlt-1 in utero. Interestingly, a high incidence of systemic hypertension has been recognized in infants with BPD, but its etiology is unclear (1). Moreover, past studies have identified an increased risk of systemic vascular dysfunction in offspring of mothers with PE, but the mechanism remains unknown (33, 35, 58, 70, 72). As shown in a previous study, neonatal treatment with truncated sFlt-1 impairs the growth of renal glomerular capillaries in mice (22), suggesting that renal vascular injury may contribute to systemic hypertension or LVH following disruption of VEGF signaling during development. Future studies would determine whether systemic vascular abnormalities have existed early in those with maternal PE, contributing to hypertension in infants with BPD and predisposing to cardiovascular diseases later in life.

This study provides mechanistic insights underlying the risk of preterm infants for BPD in the setting of maternal PE. In one study on preterm infants with very low birth weight, maternal PE is associated with increased BPD at 36 wk corrected age although it did not increase oxygen requirement at 28 days postnatal age (40). These findings suggest that progressive worsening of lung disease can occur later in the neonatal course of preterm infants born to preeclamptic mothers, which is a pattern observed in clinical BPD (6, 13, 34). Moreover, the present study demonstrates that the fetal environment of PE is sufficient to cause sustained and late pulmonary structural abnormalities characteristic of human BPD, despite the absence of adverse postnatal stimuli, such as hyperoxia, mechanical ventilation, infection, or related problems. Collectively, these findings suggest the need for new strategies to prevent BPD, of which the incidence seems to have reached a plateau in the very premature infants (59, 66). Future research is required to investigate whether early intervention, before those high-risk infants become symptomatic for clinical deterioration, may rescue the preterm lung that has been injured before birth.

A potential limitation of this rat model is the lack of direct placental production of sFlt-1 to more comprehensively reproduce the fetal environment of PE. We speculate that it explains why the sFlt-1 rats did not have signs of intrauterine growth restriction (IUGR), which is a common complication of PE. On the other hand, the current study demonstrates impaired neonatal lung growth in the absence of reduced birth weight or decreased postnatal somatic growth, indicating that impaired lung growth following intra-amniotic sFlt-1 treatment is not due to IUGR or neonatal malnutrition in this model. Moreover, the present study highlights that excess intra-amniotic sFlt-1 per se is sufficient to impair lung VEGF signaling at birth.
amniotic sFlt-1 after single injections in this rat model. In patients diagnosed with PE, the levels of sFlt-1 in amniotic fluid are rather relatively steady, since sFlt-1 is constantly overproduced in the placenta.

We conclude that intra-amniotic sFlt-1 treatment during late gestation impairs VEGF signaling and increases apoptosis in the newborn rat lung, which is followed by persistent reductions in alveolarization and pulmonary vascular growth and the subsequent development of biventricular hypertrophy during infancy. We speculate that disruption of VEGF signaling in utero due to antenatal exposure of excess sFlt-1 contributes to an increased risk for BPD and the predisposition for pulmonary and systemic vascular dysfunction in infants with maternal PE.

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DISCLOSURES

Dr. Karumanchi is a co-inventor on multiple patents held by the Beth Israel Deaconess Medical Center that are related to angiogenic factors for the use in diagnosis and therapy of preeclampsia. Dr. Karumanchi has financial interest in Agamin LLC.

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