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High-dose vascular endothelial growth factor increases surfactant protein gene expressions in preterm rat lung

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Abstract

Aims: To investigate the effects of intra-amniotic vascular endothelial growth factor (VEGF) treatment on surfactant pool sizes and surfactant protein (SP) gene expressions in fetal rat lung.
Method: On the 18th day of gestation, an abdominal midline incision was performed on timed pregnant Sprague–Dawley rats and the two uterine horns were exposed. VEGF (2.5 μg or 5.0 μg) and saline were injected into the amniotic cavity of the left and right uterine horns, respectively. On the 19th day of gestation, fetuses were delivered by caesarean section.
Results: We analyzed the data between the fetuses within the same dam in each group. Mean fetal body weight and lung tissue saturated phosphatidylcholine and total phospholipids were comparable between control and VEGF-treated rats at each VEGF dosage. Lung *SP* mRNA expressions were comparable between control and VEGF 2.5 μg-treated rats. VEGF 5.0 μg treatment increased lung *SP* mRNA expressions and the values were statistically significant for *SP-B* and *SP-D* mRNAs when compared with the control rats.
Conclusions: These results suggest that VEGF might have potential therapeutic implications in enhancing fetal lung maturation.

1. Introduction

Respiratory distress syndrome (RDS) is a major cause of morbidity and mortality in preterm neonates [1]. Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen that regulates endothelial cell differentiation and angiogenesis [2,3]. Angiogenesis is necessary for alveolarization during normal lung development [4]. VEGF has been reported to increase surfactant protein (*SP*)-*A* mRNA, *SP-C* mRNA, and SP-A protein levels, and [^{3}H]choline incorporation into total phosphatidylcholine but not into disaturated phosphatidylcholine in human fetal lung explant culture [5]. Compernolle et al. found that intra-amniotic injection of VEGF improved lung maturation in mice and suggested the VEGF stimulated conversion of glycogen to surfactant phospholipids [6]. The improvement of lung maturation was demonstrated histologically by the thinning of alveolar septa

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and disappearance of PAS-positive cells. However, it is not clear whether the prevention of neonatal respiratory distress observed following intra-amniotic administration of VEGF was associated with an increase in surfactant phospholipids. Therefore, we designed this study to investigate the effects of intra-amniotic VEGF on surfactant pool sizes and surfactant protein gene expressions in fetal rat lung.

2. Materials and methods

This study was approved by the Animal Care and Use Committees at Taipei Medical University and was performed with timed pregnant Sprague–Dawley rats (vaginal smear positive, day 0; term, day 22). On the 18th day of gestation, rats were anesthetized with pentobarbital (50 mg/kg, i.p., Abbott Laboratories, North Chicago, IL, USA). An abdominal midline incision was performed, and the two uterine horns were exposed and kept moist throughout the surgery with phosphate-buffered saline (pH 7.4). The authors recorded and counted the numbers of fetus which received VEGF or saline in left or right uterine horn at experiment, respectively. Recombinant rat VEGF (2.5 μg/40 μl saline or 5.0 μg/40 μl saline, R&D Systems, Minneapolis, MN, USA) and saline were injected into each individual gestational sac of the left and right uterine horns, respectively. The uteri were returned to the abdominal cavity, and the incision was closed layer by layer. On the 19th day of gestation, all dams were anesthetized with pentobarbital (50 mg/kg, i.p.) and the fetuses were delivered by caesarean section and weighed.

Lung tissues were homogenized and extracted with chloroform–methanol [7]. Lipid extracts were treated with osmium tetroxide, and saturated phosphatidylcholine was recovered by alumina column chromatography and quantified by phosphorus assay [8,9]. Values are expressed as micromoles per gram wet lung weight.

Lung tissue was ground into a powder in liquid nitrogen, and the gene expression of *SP-A*, *SP-B*, *SP-C*, and *SP-D* was measured with reverse transcription-polymerase chain reaction (RT-PCR). Fetal rats in the opposite uterine horn served as controls for the two VEGF doses in each litter. RNA was extracted using the TRIzol reagent (Life Technologies, Paisley, UK) according to the manufacturer's instructions. The yield and purity of the isolated RNA solution were determined by A260 and A280 readings on a spectrophotometer. Reverse transcription was performed on 3 μg of RNA with an oligo (dT) primer and avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, IN, USA). The amplifications of rat SP cDNAs were similar to the method previously described [10]. The sense primers for *SP-A*, *SP-B*, *SP-C*, *SP-D*, and *β-actin* were 5′-GGA AGC CCT GGG ATC CCT GGA-3′, 5′-GTT CCA CTG CAG ATG CCA TTG-3′, 5′-GAT TAC TCG ACA GGT CCC AGG AGC CAG TTT CG-3′, 5′-ACT TCC AGA CAG TGC TGC TCT GAG GC-3′, and 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′, respectively. The antisense primers for *SP-A*, *SP-B*, *SP-C*, *SP-D*, and *β-actin* were 5′-TGG GTA CCA GTT GGT GTA GT-3′, 5′-CAT GTG CTG TTC CAC AAA CTG C-3′, 5′-TGG CTT ATA GGC GGT CAG GAG CCG CTG GTA-3′, 5′-ATA ACC AGG CGC TGC TCT CCA CAA GCC-3′, and 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′, respectively. All the primers of the target genes were designed to span introns. The amplified cDNA size generated from the set of *SP-A*, *SP-B*, *SP-C*, *SP-D*, and *β-actin* primers were 558 bp, 215 bp, 306 bp, 456 bp, and 764 bp, respectively. The cycles to perform PCR for *SP-A*, *SP-B*, *SP-C*, *SP-D*, and *β-actin* were 24, 27, 22, 24 and 26 respectively. The PCRs were carried out with the primers using the following conditions: 94 °C for 4 min followed by 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The PCR products were separated by electrophoresis on 2.0% agarose gel, stained with ethidium bromide, and subjected to densitometry using the documentation system 1000 (Biorad, Hercules, CA, USA). All densities were normalized against respective β-actin signals obtained from the same sample, and the data were expressed as the ratios of signal obtained from the mRNA of interest over the signal obtained from *β-actin* mRNA. To determine the linear range of the PCR, the intensity of the amplified products was plotted against the cycle number. Five samples were analyzed in triplicate in each group for each surfactant protein.

Results are presented as the mean ± SEM. The between-group comparisons were made using unpaired Student's *t* test. Significance was accepted at $p<0.05$.

Table 1 Effects of intra-amniotic VEGF (2.5 μg) treatment on fetal body weight, lung tissue saturated phosphatidylcholine and total phospholipids in preterm rats

	Control	VEGF (2.5 μg)	*p*
N	16	10	
Body weight (g)	3.81 ±0.07	3.59 ±0.07	0.06
Saturated phosphatidylcholine (μmol/g lung)	2.97 ±0.13	2.65 ±0.10	0.09
Total phospholipids (μmol/g lung)	13.60 ±0.44	13.36 ±0.41	0.72

Values are expressed as mean ± SEM. *N* is the number of fetuses tested.

Table 2 Effects of intra-amniotic VEGF (5.0 μg) treatment on fetal body weight, lung tissue saturated phosphatidylcholine and total phospholipids in preterm rats

	Control	VEGF (5.0 μg)	*p*
N	25	18	
Body weight (g)	2.02 ±0.08	2.05 ±0.08	0.79
Saturated phosphatidylcholine (μmol/g lung)	1.27 ±0.10	1.48 ±0.09	0.13
Total phospholipids (μmol/g lung)	8.53 ±0.69	9.59 ±0.64	0.27

Values are expressed as mean ± SEM. *N* is the number of fetuses tested.

3. Results

Two and three pregnant rats were used for 2.5 μg VEGF and 5.0 μg VEGF studies, respectively. The results were analyzed between the fetuses within the same dam.

Mean fetal body weight tended to be higher in the control when compared with VEGF 2.5 μg-treated rats ($p=0.06$) and lung tissue saturated phosphatidylcholine and total phospholipids levels were comparable between control and VEGF-treated rats (Table 1). Mean fetal body weight and lung tissue saturated phosphatidylcholine and total phospholipids levels were comparable between control and VEGF 5.0 μg-treated rats (Table 2). Mean fetal body weight, saturated phosphatidylcholine, and total phospholipids were significantly higher in the VEGF 2.5 μg-treated group as compared to the VEGF 5.0 μg-treated group ($p<0.001$, $p<0.001$, and $p<0.001$, respectively). These results indicate that pregnant dams for 2.5 μg VEGF study had a more advanced gestation. Therefore, we controlled for this by analyzing the data between fetuses within the same dam. We do not consider this gestational disparity as a major factor to influence the outcomes as higher VEGF dosage (5.0 μg) did not increase surfactant phospholipids in less mature rat lungs.

Lung *SP-A*, *SP-B*, *SP-C*, and *SP-D* mRNA expressions were comparable between control and VEGF 2.5 μg-treated rats (Fig. 1A). VEGF 5.0 μg treatment increased lung *SP-A*, *SP-B*, *SP-C*, and *SP-D* mRNA expressions and the ratios of *SP* expression over *β-actin* of the control and VEGF 5.0 μg-treated rats were 1.06, 1.17, 1.08, and 1.13 for *SP-A*, *SP-B*, *SP-C*, and *SP-D*, respectively (Fig. 1B). VEGF 5.0 μg treatment significantly increased lung *SP-B* and *SP-D* mRNA expressions ($p<0.05$).

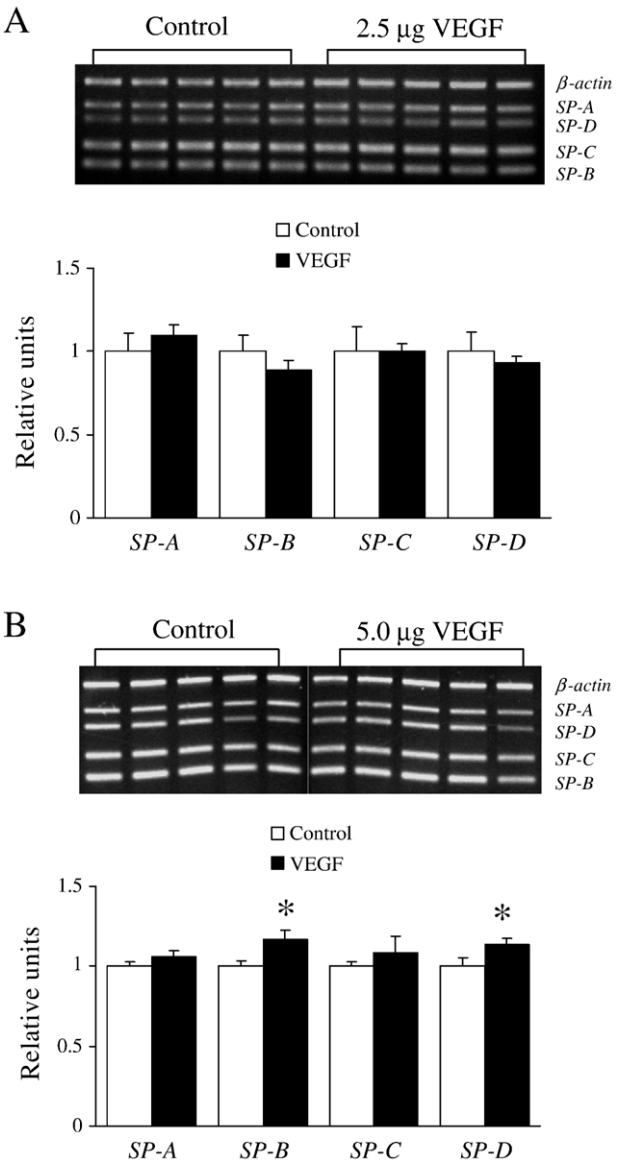

Figure 1 Effects of intra-amniotic (A) 2.5 μg VEGF and (B) 5.0 μg VEGF treatment on mRNAs encoding *SP-A*, *SP-B*, *SP-C*, and *SP-D* in the control and VEGF-treated lungs. All densities were normalized against respective β-actin signals obtained from the same sample, the data were expressed as the ratios of signal obtained from the mRNA of interest over the signal obtained from *β-actin* mRNA, and the mean normalized *SP* mRNA densitometry value for control was assigned a value of 1. The densitometry values from VEGF-treated lungs were expressed relative to controls. Lung *SP-A*, *SP-B*, *SP-C*, and *SP-D* mRNA expressions were comparable between control and VEGF 2.5 μg-treated rats. VEGF 5.0 μg treatment significantly increased *SP-B* and *SP-D* mRNA expressions. Values represent mean ± SEM for 3 separate experiments of 5 samples (*$p<0.05$ versus control group).

4. Discussion

Neonatal respiratory failure is a serious clinical problem associated with high morbidity, mortality, and costs [11,12]. The major risk factor is premature birth and the associated RDS. The pathophysiology of RDS is the immature lung structure and a deficit of surfactants. VEGF has been reported to enhance lung maturation [5,6]. However, there has been no direct evidence that VEGF increases surfactant phospholipids in fetal lung tissue. Therefore, we investigated the effects of intra-amniotic VEGF treatment on surfactant pool sizes and surfactant protein genes expression in preterm rat lung, which have been shown to be a suitable model for the study of acute neonatal lung disease [13].

Pulmonary surfactant stabilizes the lung by producing a surface-active monolayer that reduces the surface tension at the air-liquid interface of the terminal airways. Lung alveoli are lined with surfactant, which prevents their collapse on expiration and which is essential for normal postnatal lung function. A deficiency in surfactants is central to the pathophysiology of RDS [14]. Therefore, we measured the saturated phosphatidylcholine content in fetal lung tissue because it is the major surface tension-lowering component of the surfactant and makes up approximately 45% of the lung surfactant by weight. In this study, we found that intra-amniotic VEGF treatment at a dosage of 2.5 μg and 5.0 μg did not increase saturated phosphatidylcholine or total phospholipids levels in fetal rat lungs when compared with control rats, these indicate that the effect of VEGF on surfactant phospholipids was dosage independent. These results appear to be consistent with the previous *in vitro* findings that recombinant human VEGF (50 ng/ml) failed to increase saturated phosphatidylcholine and total phospholipid content of lamellar body isolated from cultured type II cells [15]. However, these are contradictory to the findings of Compernolle et al., who found that intra-amniotic injection of recombinant human VEGF enhanced lung maturation in

mice [6]. The discrepancy between our and previous findings is not clear; we speculated that it might be due to different dosage of VEGF. It is unlikely that recombinant rat VEGF in rat was not as effective as recombinant human VEGF in mice because higher VEGF dosage increased *SP-B* and *SP-D* mRNA expressions in this study and it has been shown to increase microvascular network on rat dorsal skin [16].

The mechanism by which intra-amniotic injection of VEGF can enter fetal lungs and increase surfactant synthesis is not clear. Mammalian fetal lungs secrete large amounts of fluid that may contribute to amniotic fluid and the fluid may enter the trachea during episodes of fetal breathing activity [17]. Illia et al. demonstrated that labeled surfactant injected intra-amniotically was aspirated into the guinea pig lungs [18]. Based on these studies, the authors speculate that the effect of VEGF on lung surfactant synthesis is due to its aspiration into the lung and direct contact with lung epithelium.

Four lung-specific proteins (SP-A, SP-B, SP-C, and SP-D) have been found to be associated with the surfactant [19]. Surfactant proteins are required both for the transition between lamellar bodies and tubular myelin, and for the spreading of tubular myelin components to the surface film. Previous studies of VEGF effects on *SP* expression were not consistent. Brown et al. reported VEGF increased *SP-A* mRNA, *SP-C* mRNA, and SP-A protein levels in human fetal lung explant culture [5]. Raoul et al. reported higher dosage of VEGF enhanced *SP-B* expression in isolated fetal type II cells [14]. Compernolle et al. reported that VEGF enhanced *SP-B* and *SP-C* gene expressions in isolated adult type II cells [6]. However, the *in vivo* effects of VEGF on *SP* genes expression in the fetal lung are unclear. In the present study, 2.5 μg VEGF had no effects on lung *SP* mRNA expressions; whereas 5.0 μg VEGF increased *SP* mRNA expressions and the values were statistically significant for *SP-B* mRNA and *SP-D* mRNA. These results suggest that VEGF might have potential therapeutic implications in enhancing fetal lung maturation.

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