Original Paper

Impaired VEGF Signaling in Lungs with Hypoplastic Esophageal Atresia and Effects on Branching Morphogenesis

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Key Words

VEGF • Flt-1 • Esophageal atresia • Fetal lung development • Branching

Abstract

Background/Aims: Patients with esophageal atresia (EA) and tracheoesophageal fistula (TEF) often suffer chronic respiratory tract disease. We previously reported that primary lung maldevelopment caused by deficient branching of embryonal airways in experimental EA-TEF was induced by Adriamycin. In this study, we investigated the Vascular endothelial growth factor (VEGF) pathway in the developing lung in an EA-TEF rat model. We further analyzed the effect of recombinant VEGF treatment *in vitro* on branching morphogenesis of embryo lungs in experimental EA-TEF. **Methods:** Pregnant rats received either Adriamycin or vehicle on E7, E8 and E9. Lungs were recovered at E15, E18 and E21. Expression of VEGF and receptors (Flk-1 and Flt-1) were assessed by quantitative PCR, immunohistochemistry and immunoblotting. E13 lungs were cultured for 72 hours with 50 ng/mL of recombinant rat VEGF in serum-free medium. The rates of increase in bud count and airway contour were evaluated. **Results:** Our results showed a significant downregulation of VEGF during pseudoglandular and canalicular stages. In contrast, there were significantly higher levels of the Flt-1 receptor in the canalicular stage, which may represent a compensatory response to decreased VEGF. However, both variables returned to normal levels at the saccular stage. Exogenous VEGF treatment enhanced hypoplastic lung growth, evidenced by the increase in bud count and airway contour. **Conclusions:** A VEGF signaling defect possibly plays an important role in defective embryonic airway branching. Additionally, VEGF treatment may accelerate lung growth in EA-TEF lungs.

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Introduction

Esophageal atresia (EA) is the most frequent anomaly of the esophagus and is characterized by complete discontinuity of the esophagus with or without an abnormal connection between the trachea and the distal esophagus (tracheo-esophageal fistula,

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TEF). EA affects about 1 in every 4099 births worldwide [1]. With current survival rates approaching 90% [2], long-term sequelae have become the focus of attention.

Persistent respiratory symptoms are commonly observed in children with EA, including bronchitis, brassy cough, chronic cough, pneumonia and wheezing [3-8]. Experimental models and human findings have demonstrated that developmental abnormalities of tracheal structure and innervation or lung hypoplasia might account for these sequelae beyond childhood [9-12]. In agreement with other reports, we previously reported that the lungs were hypoplastic in rats with experimental EA-TEF due to defective embryonal airway branching. These findings prompted us to unravel the mechanisms underlying the defective lung development in EA-TEF patients.

Vascular endothelial growth factor (VEGF, also designated VEGF-A), a potent mitogen and angiogenic factor, is considered to play multiple crucial roles in lung development. It exerts its functions through two high-affinity tyrosine kinase receptors, designated VEGF receptor 1 (VEGFR-1; alias FLT-1) and VEGFR-2 (alias KDR/ FLK-1). Various studies have suggested that the VEGF pathway is critical for normal lung development. Studies by Del Moral et al. point out that VEGF signaling plays a key functional role in early embryonic lung epithelial and endothelial crosstalk and branching morphogenesis [13]. Disruption of VEGF-VEGFR signaling with the VEGFR_s inhibitor could not only reduce pulmonary arterial density but also lead to reduced alveolarization and immature lung formation. These results suggest that impaired VEGF signaling impacts vascular development as well as abnormal airway growth [14-16]. The importance of VEGF for normal lung development is also highlighted by the fact that VEGF levels in tracheal fluid and lung tissue from infants dying from bronchopulmonary dysplasia are markedly reduced [17].

These facts led us to speculate that disruption of VEGF-receptor signaling might contribute to defective branching morphogenesis in EA-TEF. The objective of this study was to determine the expression of VEGF and its receptors in the developing lung using a rat model of EA-TEF induced by Adriamycin and to analyze the effect of exogenous VEGF on branching morphogenesis and epithelial differentiation of embryo lungs.

Materials and Methods

Animals

All procedures/protocols were approved by the Animal Research Committee of China Medical University (Shenyang, China). Timed-pregnant Wistar rats received three intraperitoneal injections on embryonic days E7, E8 and E9, each with 1.75 mg/kg Adriamycin (ADR, Haizheng Pharmaceutical Co. Ltd, Taizhou, China) in saline, as described previously [18]. This induces EA-TEF in 79.4% of fetuses [18]. Control rats received an equivalent volume of saline. A subset of rat dams were killed by injection of potassium chloride on E15, E18 and E20. Embryo lungs were removed and dissected free from the trachea. Another subset of dams were killed on E13 and fetal lung rudiments were collected aseptically for explant studies as described below. The lungs of ADR-treated animals were collected irrespective of the presence or absence of EA/TEF since they share similar weight, histology and branching mode of explants, as evidenced by our previous research. The experimental design is summarized in Fig. 1.

Morphometry

Immunohistochemical (IHC) studies were performed on fixed lungs. As described previously [18], after tracheal cannulation, 4% paraformaldehyde was injected at < 10 cm/H₂O for alveolar distention and fetal lungs were harvested, fixed and embedded in paraffin. IHC staining was performed on 4 µm sections using standard techniques with anti-VEGF (19003-1-AP, Proteintech, Rosemont, Illinois, USA) and anti-Flt-1 (ab32152, Abcam, Cambridge, Massachusetts, USA) antibodies.

RNA extraction and RT-PCR

Total RNA was isolated from snap-frozen lungs with TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. RNA concentration was determined in duplicate using a

Fig. 1. Diagram of experimental design. Timed-pregnant Wistar rats received three intraperitoneal injections of 1.75 mg/kg Adriamycin or saline on E7–E9. A subset of rat dams were sacrificed on E13 and embryo lung rudiments were collected and cultured in DMEM/F12 medium with or without recombinant VEGF164 for 72 hours. Another subset of dams were sacrificed on E15, E18 and E20 and fetal lungs were collected for IHC, western blot and quantitative PCR studies.

Table 1. Primers for quantitative PCR. β -actin was used as a housekeeping gene for normalization of data

Gene name	Accession Number	Primer Sequences	Size(bp)
<i>Vegf</i>	AF215726	F: 5- CAGCTATTGCCGTCCAATTGA -3 R: 5- CCAGGGCTTCATCATTGCA-3	131
<i>Flt-1</i>	NM_019306	F: 5- TGTCTCAACTGCACCGTCAC-3 R: 5- CCGCTGCCTGATAGATGCTCTC-3	100
<i>Flk-1</i>	NM_013062	F: 5- TAGCACGACAGAGACTGTGAGGT-3 R: 5- TGAGGTGAGAGAGATGGGTAGG-3	113
β -Actin	NM_031144.3	F: GGAGATTACTGCCCTGGCTCCTA R: GACTCATCGTACTCCTGCTTGCTG	190

spectrophotometer (Nano Vue; GE Healthcare, Buckinghamshire, England, UK). Two micrograms of total RNA was reverse transcribed using the M-MLV First Strand kit (C28025-032, Invitrogen). To minimize variation in the reverse transcription reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. RT-PCR was performed on a LightCycle480 instrument (Roche, Basel, Switzerland). Quantitative PCR was used for the analysis of VEGF, Flt-1 and Flk-1 mRNA levels in the fetal lung. cDNA was amplified using a LightCycler® 480 SYBR Green I Master instrument (Roche, Mannheim, Germany) in a 20 μ l reaction solution with specific primers for pan-VEGF, Flt-1 and Flk-1 (Table 1). Specificity of PCR products were confirmed by analysis of the dissociation curve. In addition, the expected amplicon size and the absence of nonspecific products were confirmed by analysis of the PCR products on 2% agarose gels, subsequently stained with ethidium bromide and visualized under UV light. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with the housekeeping gene β -actin.

Protein assay and Immunoblotting

Immunoblotting was used to detect the presence of VEGF and Flt-1 protein in rat embryo lungs at each time point. Frozen lungs were washed with ice-cold PBS and homogenized in RIPA lysis buffer (50

mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% sodium deoxycholate, 1 mM PMSF, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 0.5 mM Na_3VO_4). Lysates were centrifuged at $12,000 \times g$ for 20 minutes at 4°C and supernatants collected. Total protein content was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, Massachusetts, USA), as described by Towbin et al. [19]. The PVDF membrane was blocked with 5% nonfat milk and immunoprobed with anti-VEGF and anti-Flt-1 antibody at 4°C overnight, followed by horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hours at room temperature. Visualization of antigen-antibody complexes were accomplished using ECL plus reagent (GE Healthcare, Piscataway, NJ, USA). Quantity One software (Bio-Rad) was used to analyze optical density (OD) of protein bands. Relative expression quantity of target protein was illustrated as the percentage of the OD of target protein adjusted with corresponding β -actin OD. Experiments were performed at least three times for each antibody.

In vitro lung explant studies

On the 13th day of gestation, embryos were aseptically removed by cesarean section from pregnant rats under chloral hydrate anesthesia. As previously reported [18], fetal lung rudiments were collected aseptically, transferred to Costar-Transwell cells (Corning, Corning, NY, USA) and cultured at the air-liquid interface in serum-free DMEM/F12 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The explants were placed in humidified incubators at 37°C with an atmosphere of air plus 5% CO_2 and cultured for 72 hours. For analysis of VEGF effects, lung explants were treated daily with recombinant rat VEGF (Gibco, Invitrogen) at 50 ng/ml final concentrations from time 0 to 72 hours. The lung cultures were divided into 3 groups ($n = 8$): Group I, controls; Group II, ADR without VEGF; Group III, ADR with VEGF. The explant lungs were photographed daily on an inverted phase-contrast microscope and the digitized lung images were blindly analyzed for lung bud count and airway contour using Image Pro-Plus software (Media Cybernetics, Washington, DC, USA). The following formula was used to calculate an increase in lung bud count rate: Increased rate = (Bud count at each time point – bud count at time zero) / bud count at time zero, the increase in airway contour were calculated in the same way.

Statistical analysis

Data are presented as the mean $\pm$ SEM. Statistical analyses were performed with GraphPad Prism 5 (GraphPad software Inc, San Diego, CA, USA). Between-group differences were tested with nonparametric methods (Mann-Whitney test for two groups and Kruskal-Wallis test for more than two groups). When appropriate, parametric methods (ANOVA or Student's *t* test) were preferred. A statistical probability of $P < 0.05$ was considered significant.

Results

VEGF expression in the developing lung

By IHC, VEGF protein was mainly localized to the bronchial epithelial cells and type 2 pneumocytes. Positive VEGF staining was also noted in the mesenchyme. No difference was observed in the localization pattern of VEGF in normal and hypoplastic lungs at all three developmental stages (Fig. 2). We did not calculate the grey value because evaluation of staining intensity by IHC is subject to observer variability.

We proceeded to assess the expression of VEGF and receptors by quantitative PCR and/or western blot, as the latter two methods are more quantifiable and reproducible. The results of quantitative PCR experiments indicated that both groups shared similar developmental expression patterns of pan-VEGF mRNA. From E15 to E18, pan-VEGF mRNA levels continued to increase, but at E15 and E18 there was a tendency toward lower levels in hypoplastic lungs compared to control lungs. Although, at E18, the change did not achieve statistical significance. For parallel protein level analyses, we also used β -actin as the loading control. Similar to our mRNA data, we observed decreases in VEGF protein levels in

Fig. 2. Effect of ADR on VEGF expression in fetal lungs. (A, B) Pan-VEGF mRNA levels in the fetal lungs of both groups were determined by quantitative PCR and specificity of PCR products were confirmed by visualization on 2% agarose gels. Data are expressed relative to the control at E15, after normalization with β -actin mRNA levels. Results are presented as the mean $\pm$ SEM. * P < 0.05, ADR-treated vs. control at the same time point, inter-group; # P < 0.05, E18 or E21 vs. E15, inner-group. (C–F) Representative photomicrographs of IHC staining for VEGF in the lung sections from controls (C, E) and ADR-treated rats (D, F) on E18 (upper panel) and E21 (lower panel) (Original magnification 400 $\times$, scale bar = 100 μ m). VEGF was mainly expressed in the airway epithelium and also detectable in some mesenchymal cells (black arrowhead). (G, H) Representative immunoblots and densitometric analysis of VEGF protein in the fetal lungs at E15 and E18. Results were normalized relative to the expression of β -actin. * P < 0.05 vs. control at the same time point.

hypoplastic lungs at E15 and E18 compared to controls, whereas protein levels restored to normal at E21.

VEGF receptors in the developing lung

Positive immunoreactivity for Flt-1 was found in mesenchymal cells, in vascular structures of the mesenchyme as well as in the luminal airway epithelium. In ADR-treated lungs, Flt-1 mRNA showed a similar expression trend to that of normal lungs of equivalent stage. However, Flt-1 mRNA was significantly increased in hypoplastic lungs compared with control lungs at E18 (P < 0.05; Fig. 3). Similar to our mRNA data, we observed increased Flt protein levels assayed by western blot. In contrast, ADR did not induce an alteration in

Fig. 3. Temporal expression of VEGF receptors in the ADR treated lung. Flt-1 (A) and Flk-1 (B) mRNA levels in developing lungs were determined by quantitativePCR and results are expressed relative to the control at E15. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, ADR-treated vs. control at the same time point, inter-group; # $P < 0.05$, E18 or E21 vs. E15, inner-group. (C–F) Immunostaining for Flt-1 protein in embryo lung sections from controls (C, E) and ADR-treated rats (D, F) at E18 (upper panel) and E21 (lower panel). Flt-1 was widespread in the mesenchyme and luminal airway epithelium (black arrowhead) (Original magnification 400 $\times$, scale bar = 100 μ m). (G, H) Representative immunoblots and densitometric analysis of Flt-1 protein expression in the fetal lungs at E18. Results were normalized relative to the expression of β -actin. * $P < 0.05$, vs. control at the same time point.

(G, H) Representative immunoblots and densitometric analysis of Flt-1 protein expression in the fetal lungs at E18. Results were normalized relative to the expression of β -actin. * $P < 0.05$, vs. control at the same time point.

Flk-1 mRNA levels. Both groups shared a similar trend; slightly declining levels at E18 and reaching highest levels at E21.

Effect of VEGF on branching morphogenesis in lung explants

In order to unravel the potential role of VEGF in EA lung development, functional studies were performed using lung explant cultures. ADR EA and ADR-no EA lungs were not divided

Fig. 4. Effect of VEGF treatment on branching morphogenesis of fetal lung explants. E13 lungs were treated with VEGF164 (50 ng/ml) for 72 hours. Terminal buds counts (A, B), explant surface (C, D) and perimeter (E, F) were significantly reduced in the ADR-treated group compared with controls at all time points. Compared with the untreated lung, VEGF treatment markedly increased airway branching morphogenesis and terminal bud count. (G, H) Representative photographs illustrate the marking of the airways for measurement of airway contour and terminal bud count. Results are presented as the mean $\pm$ SEM. * $P < 0.05$, vs. control at the same time point; # $P < 0.05$, ADR-VEGF vs. ADR at the same time point.

due to their similar weight, histology and branching mode of explant [18]. As we previously reported, ADR exposure led to deficient airway branching evidenced by a marked decrease in terminal bud count at all time points, though they shared the similar increase rate as controls (Fig. 4). However, with VEGF treatment, the terminal bud count in hypoplastic lungs

was increased in a remarkably accelerated fashion and restored to normal levels after 48 hours of culture. The same accelerated pattern was also seen in airway contour. However this increasing trend was less remarkable, so the surface and perimeter of the explants in the ADR-VEGF group were always smaller than those of the control explants at the same time point.

Discussion

Survivors of EA-TEF often suffer from chronic respiratory tract disease characterized by hoarse cough, repeated pneumonia or bronchiectasis and chronic bronchitis [20–22]. Peptic bronchitis, gastro-esophageal reflux or fistula stump account in part for these sequelae, but defects in the airway or lung parenchyme, or lung hypoplasia might also play a role [12, 23, 24]. We previously reported deficient embryonal lung branching and lung hypoplasia in fetal rats with EA-TEF [18]. Although several pathways (e.g. Shh, FGF10) are reported to have been altered in the lungs of fetal rats with EA-TEF [25, 26], the precise mechanisms of impaired airway branching are still unclear.

Rat lung development is divided into five distinct stages: embryonic, pseudoglandular, canalicular, saccular and alveolar [27]. The genetic program of fetal lung development is tightly regulated and complex, and can be influenced by epigenetic and environmental factors during the period of development. Several crucial players of normal lung morphogenesis have been already identified, mostly belonging to the hedgehog, wingless, transforming growth factor- β , fibroblast growth factor and VEGF signaling pathways [28–31].

VEGF belongs to the platelet-derived growth factor family. As a potent angiogenic factor and mitogenic growth factor, it has been shown to play multiple critical roles in pre- and postnatal lung development [32]. Temporo-spatial alteration of VEGF expression can yield a broad spectrum of effects on lung branching [33]. Targeted deletion of VEGF-A, Flk-1, or Flt-1 further demonstrated the importance of these signaling pathways in lung development [34–36].

In this study, we investigated VEGF and its receptors in the developing lung of experimental EA-TEF rats induced by Adriamycin, and evaluated the morphologic effect on hypoplastic lungs following VEGF treatment. Our results demonstrated a significant downregulation of VEGF during pseudoglandular and canalicular stages, which is consistent with the underdevelopment of EA lungs. In the canalicular stage, there was a significantly higher level of the receptor Flt-1, which may represent at least part of the compensatory response for decreased VEGF in an effort to restore normal growth. However, both of these variables returned to normal at the saccular stage (E21), whereas lung weight and branching still decreased in comparison with controls. These results indicate that deficient lung branching is not exclusively due to a disturbed VEGF pathway.

Earlier studies indicated that exogenous VEGF stimulates the growth and differentiation of lung epithelial cells and surfactant production in fetal lung explants of humans and rats [37, 38]. More recently, evidence for the stimulatory effects of VEGF administration on structural maturation of the lung in both the normally grown and placentally restricted fetus has been described. [39]. However, to date, little is known about how exogenous VEGF affects branching morphogenesis of embryonal lungs in the EA model. Through *in vitro* experiments, the present study is the first to show that exogenous VEGF treatment enhanced hypoplastic lung growth, as evidenced by the increase in bud count and airway contour. This is in accordance with previous reports on the branching effect of exogenous VEGF on nitrofen-exposed lungs [40].

In summary, our findings suggest that disrupted molecular signaling in lung morphogenesis involving VEGF may result in impaired airway branching with consequent hypoplastic lungs. Furthermore, treatment with exogenous VEGF further enhanced branching and distal structural complexity of lung explants after maternal Adriamycin exposure. However, the mechanism of branching acceleration and cell differentiation by

VEGF remains unclear. These findings invite us to explore whether recombinant VEGF may provide an effective strategy to correct defective lung development in EA-TEF patients.

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Disclosure Statement

The authors declare that they have no competing interests.

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