Decreased Expression Levels of Leukemia Inhibitory Factor and Its Receptor During Airway Branching Morphogenesis in Nitrofen-Induced Hypoplastic Lungs

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ABSTRACT

Purpose: Pulmonary hypoplasia (PH) remains a major therapeutic challenge associated with congenital diaphragmatic hernia (CDH). Leukemia inhibitory factor (LIF) and its receptor (LIFR) play an essential role in airway branching morphogenesis in developing fetal lungs. With a significant increase just before birth, high LIF expression level has been demonstrated in the pulmonary epithelium, whereas LIFR is mainly expressed in the surrounding mesenchyme. Furthermore, LIF-deficient fetuses exhibit PH with defective airway tissue. We hypothesized that pulmonary LIF and LIFR expression levels are decreased during lung branching morphogenesis in nitrofen-induced PH.

Methods: Timed-pregnant rats received either nitrofen or a vehicle on gestational day 9 (D9). Fetuses were harvested on D15, D18 and D21, and dissected lungs were divided into control and nitrofen-exposed groups (n = 12 per time point and group). The pulmonary expression levels of LIF and LIFR were analyzed by quantitative real-time polymerase chain reaction. Immunohistochemical staining of LIF and LIFR was performed to evaluate protein expression and localization in branching airway tissue.

Results: Relative mRNA expression levels of LIF and LIFR were significantly reduced in the lungs of nitrofen-exposed fetuses on D15 (0.21 ± 0.13 vs 0.49 ± 0.19; p < 0.05 and 0.24 ± 0.13 vs. 0.36 ± 0.12; p < 0.05), D18 (0.11 ± 0.07 vs. 0.49 ± 0.41; p < 0.05 and 0.10 ± 0.03 vs. 0.16 ± 0.03; p < 0.05) and D21 (0.13 ± 0.04 vs. 0.27 ± 0.05; p < 0.05 and 0.18 ± 0.03 vs. 0.34 ± 0.12; p < 0.05) compared with controls. LIF immunoreactivity was markedly diminished in the distal airway epithelium, whereas LIFR expression level was decreased in mesenchymal cells surrounding terminal bronchioles and alveoli on D15, D18 and D21 compared with controls.

Conclusion: Decreased pulmonary LIF and LIFR expression levels may disrupt epithelial-mesenchymal interactions during lung branching morphogenesis and cause PH in the nitrofen-induced CDH model.

Key words: leukemia inhibitory factor, leukemia inhibitory factor receptor, pulmonary hypoplasia, nitrofen, congenital diaphragmatic hernia

I INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a common and life-threatening malformation that occurs in 1 in 2000 to 3000 live births¹. Pulmonary hypoplasia (PH), which is
characterized by immature lung development and impaired alveolarization, is considered to be one of the major contributors to high morbidity and mortality in newborn infants with CDH. Despite recent advances in neonatal intensive care and postnatal treatment strategies, PH remains a major challenge associated with CDH.

Teratogenic rodent models of CDH have a long history and are widely used for investigating the pathogenesis of CDH-associated anomalies in the lung. Nitrofen-induced CDH has been widely used as an experimental model as the timing of the diaphragmatic insult and PH are similar to the human situation. When nitrofen is administered to pregnant rats on gestational day 9 (D9), approximately 70% of the offspring show CDH and 100% of the offspring have PH. Although the pathophysiological mechanisms of PH in CDH have been extensively studied, the exact molecular basis of the underlying structural abnormalities in nitrofen-induced PH remains unclear.

Developing fetal lungs depend on specific cross-talk interactions between the epithelium and the mesenchyme during airway branching morphogenesis. Disrupted epithelial-mesenchymal interactions during lung branching morphogenesis have recently been shown to contribute to the development of PH in rodents with nitrofen-induced CDH. Leukemia inhibitory factor (LIF) is a member of the family of glycoprotein 130-type cytokines. LIF and its subunit receptor (LIFR) play an essential role in airway branching morphogenesis in developing fetal lungs. With a significant increase just before birth, high LIF expression level has been demonstrated in the pulmonary epithelium, whereas LIFR is mainly expressed in the surrounding mesenchyme. Furthermore, LIF-deficient fetuses exhibit PH with defective airway tissue similar to the phenotype seen in human CDH.

We designed this study to investigate the hypothesis that pulmonary LIF and LIFR expression levels are decreased during lung branching morphogenesis in nitrofen-induced PH.

II METHODS

1. Animals, drugs and experimental design

After obtaining approval (REC668b) from the local research ethics committee, pathogen-free adult Sprague-Dawley rats were mated overnight, and the presence of spermatozooids in the vaginal smear was considered as a proof of pregnancy. On gestational day 9 (D9), animals received either 100 mg of nitrofen (WAKO Chemicals GmbH, Neuss, Germany), dissolved in 1 ml of olive oil, or a vehicle alone. Fetuses were harvested by cesarean section on selected time points, namely, D15, D18 and D21. All fetal lung samples were dissected under a stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) and divided into control and nitrofen-exposed groups (n = 12 per time point and group). Specimens were either stored in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) for RNA isolation or fixed in 10% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc, Heidelberg, Germany) for histologic processing.

All animal procedures were performed following current guidelines for the management and welfare of laboratory animals and were approved by the Department of Health and Children (Ref. B100/4378) under the Cruelty to Animals Act, 1876 (as amended by European Communities Regulations 2002 and 2005).

2. Total RNA isolation and complementary DNA synthesis

Total RNA was isolated from snap-frozen lung specimens by the acid guanidinium thiocyanate-phenol-chloroform extraction method using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocol. Total RNA was quantified spectrophotometrically (NanoDrop ND-1000 UV-Vis® Spectrophotometer, Wilmington, USA), and the prepared RNA solutions were stored at –20°C. cDNA was synthesized using a Transcript High Fidelity cDNA Synthesis Kit® (Roche Diagnostics, Grenzach-Whylen, Germany) in accordance with the manufacturer’s protocol. All cDNA samples were stored at 4°C until further use.

3. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed using a LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer’s protocol. Gene-specific primer pairs used in this study are listed in Table 1. After the initialization phase at 95°C for 5 min, 55 amplification cycles were carried out. Each cycle included an initial denaturation step at 95°C for 10 sec, an annealing step at 60°C for 15 sec and an elongation step at 72°C for 10 sec. The final elongation temperature was 65°C for 1 min. The relative mRNA expression levels of LIF and LIFR were measured with a Light Cycler® 480 instrument (Roche Diagnostics, West Sussex, UK) and gene expression levels were
4. Immunohistochemistry and light microscopy

Following fixation in 10% PFA, fetal lung samples collected at each time point were paraffin-embedded, sectioned at a thickness of 5 μm, and mounted on polylysine-coated slides. Tissue sections were deparaffinized with xylene and rehydrated with ethanol and distilled water. All the sections were immersed in target retrieval solution (DAKO Ltd., Cambridgeshire, UK) in a microwave oven at 750 W for 15 min to prevent the masking of antigenic sites. After cooling for 10 min, Peroxidase Block® (DAKO, Cambridgeshire, UK) was used for 5 min to block endogenous peroxidase activity. The sections were incubated at 4°C overnight with affinity-purified goat anti-LIF (sc-1336; 1:50) and rabbit anti-LIFR (sc-659; 1:50) antibodies (Santa Cruz, Biotechnology, Inc., Heidelberg, Germany). On the next day, the sections were treated with the corresponding horseradish peroxidase-labeled donkey anti-goat (sc-2033; 1:100) (Santa Cruz, Biotechnology, Inc., Heidelberg, Germany) and anti-rabbit (K4003; 1:100) (DAKO Ltd., Cambridgeshire, UK) secondary antibodies. All the sections were developed with a diaminobenzidine-H2O2 substrate complex (DAKO Ltd., Cambridgeshire, UK) for 30 sec. Finally, the sections were counterstained with hematoxylin (Sigma-Aldrich Ltd., Arklow, Ireland) and mounted with glass coverslips using DPX (Sigma-Aldrich Ltd., Arklow, Ireland). Two investigators independently evaluated all the sections under a light microscope (Leica Microsystems AG, Heerbrugg, Switzerland).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer sequences used for quantitative real-time polymerase chain reaction.</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Sequence (5’-3’)</td>
</tr>
<tr>
<td>LIF</td>
<td>Forward ACC ATT TCC CAA CAA CGT GG</td>
</tr>
<tr>
<td></td>
<td>Reverse AGG TGA TGT TGG TCA GGG AG</td>
</tr>
<tr>
<td>LIFR</td>
<td>Forward AGA CCT CAG GCC AAC GTA AA</td>
</tr>
<tr>
<td></td>
<td>Reverse GGA ATT GAT GGA GCA CGG AC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward TTG CTG ACA GGA TGC AG AG</td>
</tr>
<tr>
<td></td>
<td>Reverse TAG AGC CAC CAA TCC ACA CA</td>
</tr>
</tbody>
</table>

normalized to the expression level of the housekeeping gene β-actin. All experiments were run in duplicate for each sample and primer pair.

4. Immunohistochemistry and light microscopy

Following fixation in 10% PFA, fetal lung samples collected at each time point were paraffin-embedded, sectioned at a thickness of 5 μm, and mounted on polylysine-coated slides. Tissue sections were deparaffinized with xylene and rehydrated with ethanol and distilled water. All the sections were immersed in target retrieval solution (DAKO Ltd., Cambridgeshire, UK) in a microwave oven at 750 W for 15 min to prevent the masking of antigenic sites. After cooling for 10 min, Peroxidase Block® (DAKO, Cambridgeshire, UK) was used for 5 min to block endogenous peroxidase activity. The sections were incubated at 4°C overnight with affinity-purified goat anti-LIF (sc-1336; 1:50) and rabbit anti-LIFR (sc-659; 1:50) antibodies (Santa Cruz, Biotechnology, Inc., Heidelberg, Germany). On the next day, the sections were treated with the corresponding horseradish peroxidase-labeled donkey anti-goat (sc-2033; 1:100) (Santa Cruz, Biotechnology, Inc., Heidelberg, Germany) and anti-rabbit (K4003; 1:100) (DAKO Ltd., Cambridgeshire, UK) secondary antibodies. All the sections were developed with a diaminobenzidine-H2O2 substrate complex (DAKO Ltd., Cambridgeshire, UK) for 30 sec. Finally, the sections were counterstained with hematoxylin (Sigma-Aldrich Ltd., Arklow, Ireland) and mounted with glass coverslips using DPX (Sigma-Aldrich Ltd., Arklow, Ireland). Two investigators independently evaluated all the sections under a light microscope (Leica Microsystems AG, Heerbrugg, Switzerland).

<table>
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<tr>
<th>Table 2</th>
<th>Relative mRNA expression levels of LIF and LIFR in fetal rat lungs.</th>
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<tbody>
<tr>
<td>LIF</td>
<td>Control</td>
</tr>
<tr>
<td>D15</td>
<td>0.49 ± 0.19</td>
</tr>
<tr>
<td>D18</td>
<td>0.49 ± 0.41</td>
</tr>
<tr>
<td>D21</td>
<td>0.27 ± 0.05</td>
</tr>
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*p < 0.05 vs Control.

5. Statistical analysis

All numerical data are presented as means ± standard error of the mean. Differences between two groups were tested using unpaired Student’s t test when the data showed a normal distribution or Mann-Whitney U test when the data deviated from the normal distribution. Statistical significance was accepted at p values of less than 0.05.

III RESULTS

1. Relative mRNA expression levels of LIF and LIFR in fetal rat lungs

The relative mRNA expression levels of LIF and LIFR were significantly reduced in the lungs of nitrofen-exposed fetuses on D15 (0.21 ± 0.13 vs. 0.49 ± 0.19; p < 0.05 and 0.24 ± 0.13 vs. 0.36 ± 0.12; p < 0.05), D18 (0.11 ± 0.07 vs. 0.49 ± 0.41; p < 0.05 and 0.16 ± 0.03 vs. 0.10 ± 0.03; p < 0.05) and D21 (0.13 ± 0.04 vs. 0.27 ± 0.05; p < 0.05 and 0.18 ± 0.03 vs. 0.34 ± 0.12; p < 0.05) compared with controls (Table 2).

2. Immunohistochemistry of LIF and LIFR in fetal rat lungs

Immunohistochemical staining of LIF and LIFR in the fetal lungs on D15, D18 and D21 was performed to evaluate whether the reduced amounts of LIF and LIFR transcripts were also reflected in decreased amounts of LIF and LIFR proteins. LIF immunoreactivity was markedly diminished in the distal airway epithelium, whereas the LIFR expression level was decreased in mesenchymal cells surrounding terminal bronchioles and alveoli on D15, D18 and D21 compared with controls (Fig. 1).

IV DISCUSSION

Lung branching morphogenesis is a fundamental mechanism during fetal lung development, which requires the expression of multiple regulatory factors17-19. The complex three-dimensional shapes derived by this process reflect equally
complex genetic interactions between branching epithelia and their surrounding mesenchyme. During airway branching morphogenesis, specific cross-talk interactions between the epithelium and the mesenchyme play an important role in normal fetal lung development. In recent years, in vitro and in vivo studies have provided new insights into the role and the influence of specific signalling receptor systems on the process of pulmonary organogenesis, demonstrating the role of growth factors and their receptors in lung development.

LIF is one of the members of the glycoprotein 130-type cytokines and regulates many cellular responses such as proliferation and differentiation. It has been reported that LIF and its subunit receptor (LIFR) are both involved in the epithelium-mesenchyme cross-talk, and LIF and LIFR are well known to play essential roles in airway branching morphogenesis in developing fetal lungs. LIF has been found to be first expressed in the bronchiolar and alveolar lung epithelium with additional mesenchymal expression at the end of gestation, whereas LIFR is first mainly expressed in the surrounding mesenchyme, but after the pseudoglandular stage, it is also observed in epithelial cells. Although a recent study using an experimental lung explant model showed that LIF and LIFR may have a potential inhibitory physiological role in fetal lung branching morphogenesis, LIF-deficient mouse fetuses exhibit PH and defective differentiation of the alveolar epithelium similar to human PH associated with CDH.

In the present study, we analyzed pulmonary LIF and LIFR expressions in the nitrofen-induced CDH model and demonstrated that pulmonary LIF and LIFR expression levels are decreased during lung branching morphogenesis. Lung branching morphogenesis is directed by epithelial-mesenchymal interaction, which requires the expression of multiple regulatory factors. The occurrences of LIF expression in the airway epithelium and LIFR expression in the mesenchyme suggest a key role for LIF in branching morphogenesis mediated by epithelial-mesenchymal interaction. Therefore, we speculate that the decreased pulmonary LIF and LIFR expression levels may interfere with epithelial-mesenchymal interaction during lung branching morphogenesis and thus contribute to PH in this model. Further studies investigating the regulatory factors of LIF and LIFR signalling pathway may provide new insights into the pathogenesis of PH in the CDH model.

**ACKNOWLEDGMENTS**

The authors report no conflicts of interest.
REFERENCES


