PULMONARY DEVELOPMENT
IN
CONGENITAL DIAPHRAGMATIC HERNIA
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PULMONARY DEVELOPMENT
IN CONGENITAL DIAPHRAGMATIC HERNIA

Longontwikkeling bij congenitale hernia diafragmatica

THESIS

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and in accordance with the decision of the Doctorate Board

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CONTENTS

chapter 1
Introduction

chapter 2
Expression of Glucocorticoid, Retinoid, and Thyroid Hormone Receptors during Human Lung Development

chapter 3
Spatial and Temporal Expression of Glucocorticoid, Retinoid, and Thyroid Hormone Receptors is not Altered in Lungs of Congenital Diaphragmatic Hernia

chapter 4
Expression of Hypoxia-Inducible Factors in Normal Human Lung Development

chapter 5
The Effect of Oxygen Concentration on Hypoxia-Inducible Factors Expression in Human Fetal Lung Explants

chapter 6
Cell Proliferation and Apoptosis in Lungs of Patients with Congenital Diaphragmatic Hernia

chapter 7
General Discussion

chapter 8
Summary
Samenvatting

Curriculum Vitae
Acknowledgements
Color Figures
INTRODUCTION

When congenital diaphragmatic hernia (CDH) was first described in the early 18th century, it was considered as a result of an opening in the diaphragm that theoretically could be easily corrected after birth by removal of the herniated viscera and subsequent closure of the diaphragm. Over the past three decades, however, it has become evident that CDH is an anomaly characterized by not only a diaphragmatic defect, but also a variable amount of pulmonary hypoplasia (PH) and lung immaturity in some cases. Apart from these features, pulmonary vascular abnormalities may occur and cause persistent pulmonary hypertension of newborn (PPHN).\(^1,2\)

Hypoplastic lungs are characterized by fewer airways and smaller air spaces, and thus the lower number of vascular generations and increased adventitia and medial thickness of pulmonary arterial walls give rise to pulmonary hypertension.\(^1,2\) Apart from the morphologic abnormalities in the pulmonary vasculature, an alternate expression of various cellular mediators, such as nitric oxide, endothelin, prostaglandins, catecholamines, and renin-angiotensin system, have been suggested to contribute to the pathogenesis of PPHN in CDH patients.\(^3,4\)

Classically, CDH is considered as a primary defect of diaphragmatic embryogenesis resulting from failure of formation or fusion of pleuroperitoneal membranes. Consequently, the abdominal organs herniate into the ipsilateral thoracic cavity when the midgut returns to the abdominal cavity around the 10th gestational week. The timing of herniation coincides with the critical period of lung development and in utero competition for space of the developing lung and the ‘abdominal viscera’ leads to pulmonary hypoplasia.\(^5\) However, more recent experimental evidence suggests that lung hypoplasia may occur independently as a result of defects in signaling pathways.\(^6\) Recently the retinoid hypothesis has gained much interest – reviewed by Clugston et al.\(^7\) – and also we have gained much greater understanding of the role of specific genes in the etiology of CDH.\(^8\) Different animal models have been used to study the natural history of CDH, but current understanding of its etiology and pathophysiology remains limited.

EPIDEMIOLOGY AND CLINICAL ASPECTS OF CDH

The incidence of CDH is approximately one in 2500 live births.\(^9\) The typical form is the posterolateral or so-called Bochdalek hernia (95%), of which 85% occur on the left side.\(^10\) With 2% or less incidence, familial cases are sporadic.\(^6\) A number of chromosomal anomalies have been described in CDH cases, including trisomy 13, trisomy 18 and tetrasomy 12P, but no single gene mutation has been identified.\(^11\) In some 40 to 50% of CDH cases, associated anatomical anomalies occur, with heart and limbs defects being the most frequent.\(^10,12\)
Despite advancements in neonatal intensive care and ventilation, including inhaled nitric oxide, high frequency oscillatory ventilation and extracorporeal membrane oxygenation (ECMO) in selected infants, both morbidity and mortality in infants with CDH remain high. Hidden mortalities (in utero death, termination of pregnancy) underscore the true outcome in population-based surveys. Major factors limiting survival of infants with CDH are pulmonary hypoplasia and PPHN.

As numbers of patients even in 'high volume' centers is relatively low (15 - 20 per year) the individual treatment of a new patient with CDH should still be considered a matter of trial and error. Therapies proven to be effective in randomized controlled trials are not available. As such case selection is the major determinant of outcome and depends on:
- pre/postnatal diagnosis
- inborn – outborn
- ECMO facility yes/no
- Inclusion of long-term follow-up or evaluation at hospital discharge, etc.

Prenatal Diagnosis

The increased use of prenatal ultrasound allows antenatal diagnosis of CDH. The diaphragm can be visualized as early as in the first trimester. Its absence is indirectly evident by the presence of abdominal organs in the thoracic cavity and displacement of thoracic organs. A European study reports around 60% antenatal diagnosis of CDH, at a mean gestational age of 24 weeks. Prenatal diagnosis has raised the issue of predicting survival and outcome. Many different criteria have been proposed for prenatal imaging. Lung-to-head ratio, evidence of liver herniation or associated anomalies are widely used as prognostic factors, although still under debate. There is still no consensus, however, on the ideal determinant of postnatal outcome in infants with CDH. It would seem imperative to develop standardized prenatal criteria for prediction of postnatal outcome that allow accurate and thorough prenatal counseling and planning of treatment.

Postnatal Management

Basic approach
The philosophy of postnatal care has changed with the progress in understanding the pathophysiology of CDH. Until the 1980s, it was believed that an emergency surgical procedure aimed at reducing the herniated abdominal viscera could improve hypoxia and hypercarbia in infant with CDH. Since then, however, delayed surgery following stabilization has become the widely accepted approach to manage CDH patients resulting in case selection for surgical correction. Many therapeutic strategies are used
with the aim to reduce hypoxia, ventilator associated lung injury and pulmonary hypertension, and right-to-left shunting. High-frequency oscillatory ventilation (HFOV) is used to increase tissue oxygenation whilst minimizing volutrauma to the lung. Although not yet evaluated in randomized controlled trials, ECMO has been accepted as a method for treatment of infants with respiratory failure refractory to all other ventilatory and supportive therapies. However, HFOV and ECMO show no clear benefit over conventional respiratory care in CDH patients. 21-25 Today clinicians are aware that the different ventilatory regimens have deleterious effects on the hypoplastic lung. More centers now advocate prevention of ventilator-induced lung injury by using the ‘gentle ventilation’ concept of permissive hypercapnia, spontaneous respiration and avoidance of hyperventilation and barotraumas in CDH infants. 24-26 Exogenous surfactant therapy is widely used to improve oxygenation in premature infants but observational data from the International CDH Registry do not confirm any benefit of this therapy, neither in preterm nor in term born infants with CDH. 27,28 Today no data are available showing a primary surfactant deficiency in newborns with CDH. 29

**In Utero Management of CDH, the Ultimate Solution?**

While advances in neonatal care have to some extent improved the survival of infants with CDH, morbidity still remains significant, for example in the form of chronic oxygen dependency, gastroesophageal reflux, poor growth and developmental delay. The evolution of prenatal diagnosis offers a possibility for prenatal interventions in an attempt to promote lung growth before birth and consequently to improve the prognosis.

**Fetal surgical interventions**

Primary prenatal repair was unsuccessful in most of the severe cases (those with liver herniation) because reduction of the liver back into the abdomen caused obstruction of umbilical venous blood flow, leading to bradycardia and fetal death. Although this procedure is technically feasible, a randomized controlled trial in fetuses without liver herniation did not show a significant benefit over conventional postnatal care. 30 In view of this finding, efforts then were focused directly on treating pulmonary hypoplasia.

The observation that infants with laryngeal atresia develop enlarged hyperplastic lungs forms the basis for the PLUG approach: Plug the Lung Until it Grows. 31 Fetal tracheal ligation in CDH results in tremendous lung growth, reversal of pulmonary hypoplasia but fewer type II pneumocytes and lower alveolar phospholipid content. 32,33 A randomized controlled trial sponsored by the US National Institutes of Health (NIH) reported that fetal endoscopic tracheal occlusion failed to improve survival or morbidity rate of the fetuses with CDH compared with standard postnatal care. 34 To date the trials continue in Europe using temporary fetoscopic endoluminal tracheal occlusion with refined techniques and inclusion criteria, 17 in selected ‘liver-up’ cases without associated anatomical or chromosomal abnormalities. At present over 40 fetuses have been evaluated and
compared with cases with a proven mortality rate of over 80% (Personal communication, Deprest, Belgium).

Pharmacological intervention
Hormonal therapy
Antenatal glucocorticoid (GC) therapy was proven to accelerate pulmonary maturity and is recommended for all pregnancies between 24 and 34 weeks of gestation if premature delivery is expected. In the Nitrofen-induced CDH rat model, there is evidence that antenatal steroids improve lung maturity, reduce saccular septal thickness and increase surfactant content. However, the evidence of surfactant deficiency and lung immaturity in animals and human infants with CDH is controversial. Several reports have shown normal surfactant levels in rats with Nitrofen-induced CDH as well as in patients. Despite encouraging result in three CDH cases reported by Ford et al., a recent report by the CDH Study Group does not recommend antenatal steroid administration beyond 34 weeks of pregnancy. A planned trial evaluating the standardized use of corticosteroids in prenatally diagnosed CDH was never achieved due to the low inclusion rate and absence of Institutional Review Board approval in many centers.

The significant effect of thyroid hormone on lung development was observed in the Nitrofen-induced CDH rat model. The combined administration of thyroid hormone (T4) and Nitrofen to pregnant rats reduced the number of malformations in the offspring. Research of the role of thyroid hormone on lung development has concentrated on the later stages of lung development, particularly the regulation of surfactant production. Thyroid hormone seems to act synergistically with glucocorticoid in stimulating surfactant release; when used alone, however, it failed to show consistent stimulation effects on lung development.

Vitamin A status
The first indication of a link between vitamin A deficiency and CDH came from the observation of diaphragmatic defect in rat pups born from vitamin A deficient dams as early as 1949. Defect rates decreased when vitamin A was supplemented to the diet during mid-gestation. Some offspring of retinoid receptors (RARα/β) null-mutant mice have diaphragmatic hernia similar to that observed in Nitrofen-induced CDH rats. In the Nitrofen-induced CDH model, the antenatal administration of vitamin A to pregnant dams significantly increased survival rate and lung growth, and simultaneously decreased the incidence of CDH in the offspring. The developing diaphragm strongly expresses proteins associated with metabolism and binding of retinoids. There is now strong evidence that the retinoid signaling pathway is involved at least in the Nitrofen-induced CDH rat model. Such evidence is lacking in human CDH; only one study reported decreased blood parameters of the vitamin A status (i.e. retinol and retinoid binding protein) in newborns with CDH compared with healthy newborns. Nevertheless, retinoids are known to play a crucial role in diaphragm and lung development. Further
investigations are needed to assess the potential of prenatal vitamin A and/or derivates supplementation on outcome of CDH.

**CONCLUDING REMARKS AND AIM OF THE STUDIES**

CDH continues to be a challenge for clinicians because many questions remain unanswered so far. The variability of phenotypic expression of CDH makes it difficult to standardize treatment. Inevitably, treatment strategies have to be adapted to the individual patient. The primary goal is to reduce morbidity and mortality. Understanding the basic mechanisms of normal and abnormal lung development is mandatory to come to evidence-based therapy in CDH. Many researchers have tried to modulate growth and differentiation of either or both airways and the vasculature, either by fetal surgical interventions or by pharmacological approaches. Obviously, most of the studies are based on animal models. Up till now, human data are too limited to permit assessing the potential of new therapeutic modalities in human CDH.

In order to add to the human data, the specific aims of the studies described in this thesis are as follows:

1. Are the members of the steroid nuclear receptors superfamily differently expressed in normal and CDH human lungs during development and in the Nitrofen-induced rat model of CDH? *(Chapters 2 & 3)*
2. Are the various angiogenic factors involved in the VHL pathway expressed differently during normal human pulmonary development? *(Chapter 4)*
3. What is the effect of low oxygen concentration on the angiogenic factors VEGF, VEGFR-2, HIFs in human fetal lung explants cultures _in vitro_? *(Chapter 5)*
4. Are there any disturbances in proliferation and apoptosis in CDH human lung morphogenesis and normal lungs of different gestational ages? *(Chapter 6)*

The findings from these studies are discussed in the general discussion *(Chapter 7)* and summarized in **Chapter 8**.
REFERENCES


chapter 2

Expression of Glucocorticoid, Retinoid, and Thyroid Hormone Receptors during Human Lung Development

Prapapan Rajatapiti, Monique H.A. Kester, Ronald R. de Krijger, Robbert Rottier, Theo J. Visser, and Dick Tibboel

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ABSTRACT

Context
Although glucocorticoid hormone, thyroid hormone, and retinoic acid play important roles in fetal development, the expression of their receptors in human lung is still unknown.

Objective
The aim of this study was to investigate the ontogeny of glucocorticoid receptor (GR)α, thyroid hormone receptors (TRs), retinoic acid receptors (RARs), and retinoid X receptors (RXRs) mRNA expression in human lungs.

Design
Lungs from human fetuses and neonates (13.5 - 41 wk gestation; n = 20) as well as adults (n = 5) were analyzed by real-time PCR to monitor the ontogeny of mRNA expression for each receptor. In addition, immunohistochemistry was performed to show the cellular distribution of the different receptors.

Results
The expression of GRα, TRs, RARs, and RXRs was already detected in the earliest developmental stages analyzed. There was no significant difference in mRNA expression between developmental groups for any of the genes studied. However, for fetal and neonatal samples, there were positive correlations between gestational age and mRNA expression for RARα (r = 0.665; P = 0.001), RXRα (r = 0.444; P = 0.050), and RXRγ (r = 0.464; P = 0.039). Immunohistochemical studies showed the presence of GRα, TRs, RARs, and RXRs in the nuclei of both epithelial and mesenchymal cells, albeit more pronounced in epithelium of larger airways.

Conclusions
The detection of GRα, TRs, RARs, and RXRs expression in human lung as early as 13.5 wk gestation implies an early potential for therapeutic or toxic effects by exogenous analogs or by excess of endogenous ligands.
INTRODUCTION

In humans, lung development starts around the fourth week of gestation with the formation of two endodermally derived lung buds. The main bronchi and segmental bronchi are formed through branching morphogenesis, until the bronchial tree is completed by wk 16. The lumina of the bronchi and terminal bronchioles become larger, and vascularization is more prominent during the canalicular period (17 - 26 wk gestation). Further subdivision of bronchioles and formation of primitive alveoli occurs in the saccular period (24 - 36 wk gestation until term). In the alveolar period (from 36 wk onward), the gas exchange surface of the lung increases due to the thinning of the squamous epithelial layer, forming thin-walled alveoli. This alveolization continues after birth and is completed in childhood. 

Many factors, including hormonal, biochemical, and physical factors, have been identified that modulate growth and development of the lungs. Among these, glucocorticoids, thyroid hormone, and retinoic acid (RA) are described to influence the growth of lungs.

Glucocorticoid plays a role in the regulation of surfactant proteins and lipids during lung maturation; therefore, antenatal corticosteroids were recommended by National Institutes of Health consensus to all fetuses between 24 and 34 wk gestation at risk of preterm delivery. This will lead to an induction of surfactant production, an acceleration of lung maturation, and an increase in lung compliance. Thyroid hormone, independently or in conjunction with glucocorticoids, has been implicated in the growth and maturation of the perinatal lung. Early reports suggested that thyroid hormone concentrations in cord blood of premature infants with respiratory distress syndrome were low, relative to age and birth weight-matched controls, but these findings were not supported in later studies. Vitamin A, and its active metabolite RA, has a role in cellular differentiation via binding to its receptors. In animal studies, both the lack and excess of vitamin A during embryonic development result in congenital malformations such as infertility, anopthalmia, and lung hypoplasia. In humans, lower plasma vitamin A levels were found in prematurely born infants, especially in cases with respiratory distress or pulmonary hypoplasia associated with congenital diaphragmatic hernia.

The actions of these ligands are mediated through the activation of their receptors, which belong to a superfamily of ligand-dependent transcriptional proteins. All members of the family share a highly conserved similar modular structure with discrete functional domains for hormone binding, DNA binding, and transactivation. Through the DNA binding domain, the receptor binds to specific DNA sequences as monomers, homodimers, or heterodimers. Unlike other steroid hormone receptors, glucocorticoid receptor (GR) resides primarily in the cytoplasm of cells in the absence of ligands. Thyroid hormone receptors (TRs) are encoded by two genes (α and β), each producing several isoforms, among which TRα1 and TRβ1 act as functional receptors. Retinoid receptors are classified into two subtypes, RA receptors (RARs) and retinoid X receptors (RXRs). Each subtype comprises three major isoforms, α, β, and γ. Although 9-cis-RA binds to both RARs and RXRs, all-trans-RA binds preferably to RARs. Aside from the
classical model in which the steroid hormones elicit a transcriptional response upon binding to their cognate receptor, there appears to be a nongenomic action described for at least the glucocorticoids and the thyroid hormone. This nongenomic response occurs rapidly (within minutes) and is unaffected by inhibitors of transcription and protein synthesis. The site of the response can be at the plasma membrane, in the cytoplasm, and in cellular organelles.\(^{21-24}\)

Because the level of nuclear receptor expression determines cell sensitivities to certain hormones,\(^{25}\) the presence of these receptors in human lung has been studied previously by various techniques.\(^{26-29}\) However, the ontogeny of their expression has not been shown in a systematic way before. To elucidate their expression patterns during human lung development, we examined the expression of GRs, TRs, TR\(\beta\), RARs, and RXRs in human lung tissue, from 13.5 wk gestation until term, by real-time PCR and immunohistochemistry.

**MATERIALS AND METHODS**

**Tissue samples**

After approval of the University Ethical Committee of the experimental design and protocols, lung tissues were retrieved from the archives of the Department of Pathology, Erasmus MC, Rotterdam. Fetal and neonatal lung tissues (\(n = 20\)) were obtained from either elective termination of pregnancy or autopsies. All samples were harvested within 24 h after death. All neonatal cases died of reasons other than pulmonary abnormalities with known postconceptional age and in the absence of documented growth retardation. No gross anatomical abnormalities were documented in any of these cases on standardized pediatric pathology evaluations. The lung samples were randomly selected from either side. There were five samples for each morphological distinct developmental stage: pseudoglandular stage (13.5 - 17 wk gestation; mean, 16 wk), canalicular stage (18 - 26 wk gestation; mean, 21.8 wk), saccular stage (29 - 36 wk gestation; mean, 30.4 wk), and alveolar stage (37 - 41 wk of gestation; mean, 39 wk). Another five lung tissues from adult surgical resection specimens (normal lung tissue resected along with a tumor) were included (25 - 49 yr; mean, 38 yr). There were no pulmonary abnormalities in any of the lung specimens, especially no sign of pulmonary hypoplasia on histological screening. Tissue samples were snap-frozen and stored at \(-80^\circ\) C before RNA analysis. For immunohistochemical studies, tissues were fixed by immersion in 4\% buffered formalin and embedded in paraffin. Subsequently, 5-\(\mu\)m-thick sections were mounted on 3-amino-propyl-trioxysilane-coated glass slides (Sigma, St. Louis, MO) and processed for immunohistochemistry.

**RNA extraction and cDNA synthesis**

Total RNA was extracted from frozen lung tissues using TRIzol reagent (Invitrogen, Breda, The Netherlands), according to the manufacturer's instruction. Total RNA was quantified by measuring the absorbance at 260 nm, and the purity was checked with
260/280 nm absorbance ratio. cDNA synthesis was carried out in a reaction vol of 20 µL containing 500 ng total RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 100 ng random hexamer primer, 500 µM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 10 U ribonuclease inhibitor, and 200 U Moloney murine leukemia virus reverse transcriptase (all reagents were obtained from Invitrogen). The samples were incubated for 60 min at 37 °C, followed by incubation for 15 min at 99 °C. Negative control samples were prepared by omission of the Moloney murine leukemia virus reverse transcriptase.

Real-time quantitative PCR

Real-time PCR was performed using an iCycler IQ Real time PCR detection system (Bio-Rad Laboratories, Inc., Veenendaal, The Netherlands) and qPCR Core kit for SYBR Green I (Eurogentec, Seraing, Belgium). Gene-specific primers used in this study (Table 2.1) were designed using the sequences accessible in the NCBI Reference Sequence (www.ncbi.nlm.nih.gov/RefSeq) and Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). A total reaction vol of 25 µL contained 1X reaction buffer, 3.5 mM MgCl₂, 200 µM of each deoxynucleotide triphosphate, 250 nM of each primer, and 1 µL cDNA. The PCR thermal cycle conditions were: 10 min of initial denaturation at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 30 sec at 58 °C for annealing, 30 sec at 60 °C, and 15 sec at 75 °C. To verify the specificity of the amplified products, each PCR was followed by a melting curve analysis from 55 - 95 °C. Each sample was run as a triplicate, and mRNA of each target gene was determined simultaneously in a 96-well plate. Negative control samples and reactions mixed without cDNA templates were run in parallel.

PCR results are shown as the relative expression level of normalized samples [Δ cycle threshold (Ct)] in relation to the expression of the "calibrator" sample of 13.5 wk gestation (2−ΔΔCt), which was arbitrarily set at 100% (arbitrary value = 1). The Ct value refers to the cycle number at which the PCR plot crosses the threshold line, ΔCt is calculated by subtracting Ct value of the corresponding glyceraldehyde-3-phosphate dehydrogenase control (endogenous reference control) from the specific Ct value of the target, and ΔΔCt is obtained by subtracting the ΔCt of each experimental sample by the ΔCt of the calibrator sample. 30

Statistical analysis

Data from the real-time RT-PCR are shown as mean 2−ΔΔCt ± SEM or individually. The differences in mRNA expression between groups were analyzed with one-way ANOVA with post hoc least-significant difference test. The correlations between mRNA expression and gestational age were determined by nonparametric Spearman’s correlation. P ≤ 0.05 was considered statistically significant (see Figure. 2.2, which includes correlation coefficients only for groups for which P ≤ 0.05). All statistics were calculated using a SPSS statistical package (version 11.0; SPSS, Inc., Chicago, IL).
Table 2.1 Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’ → 3’)</th>
<th>Product size (bp)</th>
<th>Reference sequences*</th>
</tr>
</thead>
</table>
| GAPDH | Fw: TGA ACG GGA AGC TCA CTG G  
Rv: TCC ACC ACC CTG TTG CTG TA | 307 | NM_002046 |
| GRα   | Fw: GAA CTG GCA GCG GTT TTA TC  
Rv: TCT CCG GGA ATT CAA TAC TCA | 125 | NM_000176 |
| TRα1  | Fw: TCG AGC ACT ACG TCA ACC AC  
Rv: TCG ACT TTC ATG TGG AGG AA | 127 | NM_199334 |
| TRβ   | Fw: ACC AGA GTG GTG GAT TTT GC  
Rv: AAG GGA CAT GAT CTC CAT GC | 105 | NM_000461 |
| RARα  | Fw: TAC TGC CGA CTG CAG AAG TG  
Rv: CGT CAG CGT GTA GCT CTC AG | 120 | NM_000964 |
| RARβ  | Fw: GGT TTC ACT GGC TTG ACC AT  
Rv: AAG GCC GTC TGA GAA AGT CA | 129 | NM_000965 |
| RARγ  | Fw: TAC CAC TAT GGG GTC AGC  
Rv: CTG GTC ACC TTG TTG ATG AT | 119 | NM_000966 |
| RXRα  | Fw: TTC GCT AAG CTC TTG AGC  
Rv: ATA AGG AAG GTG TCA ATG GG | 113 | NM_002957 |
| RXRβ  | Fw: GAA GCT CAG GCA AAC ACT AC  
Rv: TGC AGT CTT TGT TGT CCC | 111 | NM_021976 |
| RXRγ  | Fw: ACA AGC GTC AGC GCA ACC  
Rv: GCC TCA CTC TCA GCT CGC TCT C | 121 | NM_006917 |

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase  
* Reference sequences number according to NCBI Reference sequence (RefSeq)

Immunohistochemistry

Immunohistochemistry was performed using a ChemMate Dako EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (K5007, Dako- Cytomation B.V., Heverlee, The Netherlands). In brief, after deparaffinization for 10 min in xylene and rinsing in 100% alcohol twice, slides were treated with 3% H₂O₂ in methanol for 20 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were subjected to a 15-min microwave treatment in Tris/EDTA buffer (pH 9.0) or citric acid buffer (pH 6.0). Sections were incubated for 30 min at room temperature with primary antibody (Table 2.2) in a humidified chamber. After rinsing twice with PBS/0.1% Tween 20, slides were incubated for 30 min at room temperature with HRP-conjugated dextran polymer reagent. Sections were then rinsed twice with PBS before peroxidase was detected by incubation with 3,3’-diaminobenzidine tetrachloride (1:50 dilution of ChemMate DAB+ Chromogen in ChemMate Substrate Buffer). Finally, slides were rinsed with running tap water and counterstained with hematoxylin, dehydrated through graded alcohol and xylene, and mounted. Negative controls were performed by omission of the primary antibodies.
Table 2.2 Primary antibodies for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRα</td>
<td>Rabbit</td>
<td>1:75</td>
<td>sc-1002; Santa Cruz Biotechnology, U.S.A.</td>
</tr>
<tr>
<td>TRα1α2</td>
<td>Mouse</td>
<td>1:20</td>
<td>Clone 1718; Labvision Neomarkers, CA, U.S.A.</td>
</tr>
<tr>
<td>TRβ</td>
<td>Mouse</td>
<td>1:50</td>
<td>Clone 2386; Labvision Neomarkers, CA, U.S.A.</td>
</tr>
<tr>
<td>RARα</td>
<td>Rabbit</td>
<td>1:100</td>
<td>sc-551; Santa Cruz Biotechnology, U.S.A.</td>
</tr>
<tr>
<td>RARβ</td>
<td>Rabbit</td>
<td>1:100</td>
<td>sc-552; Santa Cruz Biotechnology, U.S.A.</td>
</tr>
<tr>
<td>RARγ</td>
<td>Rabbit</td>
<td>1:200</td>
<td>sc-550; Santa Cruz Biotechnology, U.S.A.</td>
</tr>
<tr>
<td>RXRα</td>
<td>Rabbit</td>
<td>1:250</td>
<td>sc-553; Santa Cruz Biotechnology, U.S.A.</td>
</tr>
<tr>
<td>RXRβ</td>
<td>Mouse</td>
<td>1:250</td>
<td>Clone 147; Labvision Neomarkers, CA, U.S.A.</td>
</tr>
<tr>
<td>RXRγ</td>
<td>Rabbit</td>
<td>1:250</td>
<td>sc-555; Santa Cruz Biotechnology, U.S.A.</td>
</tr>
</tbody>
</table>

RESULTS

mRNA expressions

The expressions of the mRNA for GRα, TRα1, TRβ, RARs, and RXRs were detected in all the samples examined. Figure 2.1 shows average mRNA expression (mean ± SEM) of the different morphological stages. The mRNA expression of all genes studied was low in the pseudoglandular group and increased during the canalicular and the saccular stages. For RARs and RXRs, mRNA expression increased with age until adult, whereas the expression of GRα, TRα1, RARβ, and all RXRs was highest in the saccular or the alveolar group. Although there was no significant difference in mRNA expression between developmental groups for all genes studied, there were discernable differences in mRNA expression during development. When individual data points from fetus and newborn samples (13.5 - 41 wk gestation) were plotted against gestational age (Figure 2.2), there were significant positive correlations between age and mRNA expression of RARα (r = 0.665; P = 0.001), RXRα (r = 0.444; P = 0.050), and RXRγ (r = 0.464; P = 0.039). Melting curve analysis showed the generation of specific products in all PCRs, as was confirmed by running several samples on agarose gel (data not shown).

Immunohistochemistry

Immunoreactivity of GRα, TRα, TRβ, RARs, and RXRs was detected in the nuclei of cells in all samples. GRα reactivity was detected mainly in epithelial cells (Figure 2.3A). TRα was expressed in both epithelial and mesenchymal cells (Figure 2.3B), whereas TRβ reactivity was detected in the epithelial cells and endothelial cells of arteries (Figure 2.3C). Immunoreactivities of RARs and RXRs were detected in virtually all epithelial cells and in some mesenchymal cells (Figure 2.3, D–I). The expression pattern of each receptor was similar in all developmental groups. Because the immunohistochemistry was basically to demonstrate the regions and cellular distribution of the receptors, we did not attempt to quantify the staining intensity.
Figure 2.1 The ontogenic change of mRNA expression of GRα, TRα1, TRβ, RARs, and RXRs in human lungs. Samples were grouped according to their morphological stages and gestational age: pseudoglandular (13.5 - 17 wk), canalicular (18 - 26 wk), saccular (29 - 36 wk), alveolar (37 - 41 wk), and adult (25 - 49 yr); n = five per group. Data are shown as mean $2^{\Delta\Delta Ct} \pm$ SEM (as outlined in Materials and Methods). There was no significant difference in mRNA expression in all encoded genes ($P > 0.05$).
Figure 2.2 Relation between GRα, TRα1, TRβ, RARs, and RXRs mRNA expression and gestational age of 20 samples from 13.5 - 41 wk of gestation. The r-values show Spearman’s correlation coefficient with gestational age.
Immunostaining of representative samples of human fetal lung. All pictures were taken from samples of 18 weeks of gestation except for TRβ (32 wk) and RXRβ (13.5 wk). The immunoreactivity was visualized by diaminobenzidine, positive cells giving a brown color at the site of reaction. (A), GR immunoreactivity was expressed mainly in nuclei of epithelial cells. (B), TRα was detected in both epithelial and mesenchymal cells, while TRβ (C), was detected in airway epithelium and some vascular endothelial cells (arrow). Immunoreactivities of RARα (D), RARβ (E), RARγ (F), RXRa (G), RXRβ (H), and RXRγ (I) were detected virtually in all epithelial and in some mesenchymal cells. (scale bar, 50 µm)

**DISCUSSION**

The developing lung undergoes a series of complex changes both during embryogenesis and after birth. Although a number of studies were conducted to evaluate the roles of glucocorticoids, thyroid hormone, and RA in lung formation, there is limited knowledge on the ontogeny of the expression of their receptors during human lung development. In this study, we investigated the mRNA expression of GRα, TRs, RARs, and RXRs in human lung throughout gestation, using real-time quantitative PCR and immunohistochemistry. We detected mRNA and protein expression of all receptors in human lung from the earliest stage, 13.5 wk gestation, onward.

The GR is a ubiquitously expressed hormone-dependent transcription factor involved in the regulation of many physiological processes. In humans, Ballard and Ballard first reported GR mRNA expression in lungs of fetuses and neonates (age ranging from 12 -
Hormone Receptors in Developing Human Lung

43 wk gestation) using cytoplasmic binding and nuclear uptake assays. In 1990, Labbe et al. measured GR concentration in fetuses (15 - 28 wk gestation), infants, and children (2 months to 9 yr). The receptor concentration in the fetuses was high, irrespective of gestational age, and the mean concentration was significantly lower postnatally. Moreover, to evaluate the functional role of GR, several experiments with mutant mice have revealed that lung maturation was delayed in mice with low or no GR expression. The lungs of the GR null mice are condensed and hypercellular, with reduced septal thinning, leading to an increase in the airway-to-capillary diffusion distance.

Our quantitative PCR data showed a slightly different pattern, i.e. low GRα mRNA expression at the pseudoglandular stage, increasing during gestation, and lower in adults. We did not include infant specimens in our study. We found the immunohistochemical localization of the GR in the nuclei of airway epithelium and mesenchymal cells, albeit more prominent in the epithelium. This finding is consistent with previous studies performed in adult lungs, which showed GR to be localized in alveolar wall, airway epithelium, and vascular endothelial cells. Oakley et al. showed that the GRβ resides in the nucleus of cells, irrespective of hormonal binding, whereas GRα is in the cytoplasm and translocates to the nucleus upon ligand binding.

Thyroid hormone is essential for normal growth and development. Serum T₃ and T₄ are present in the fetal circulation before the onset of fetal thyroid hormone production, albeit at low levels. During the first trimester, maternal thyroid hormone is transferred into fetal circulation via the secondary yolk sac; and during the second trimester, it is transferred directly into fetal blood. Subsequently, there is an increasing contribution of thyroid hormone production by the fetal thyroid gland. The role of thyroid hormone in lung development is exerted predominantly in the late phase of lung development, with regard to the regulation of surfactant production. In rats, administration of T₃ accelerates the process of septation, resulting in a greater alveolar surface area. TRα1 and TRβ act as T₃-dependent transcription factors. TRs can bind to the DNA as homodimers or as heterodimers with other nuclear receptors, such as RXRs. Increases in TR binding capacity and occupancy by T₃ during lung development in rabbits suggest a physiological role of thyroid hormone in lung development. Little is known about the cellular pattern of TR in the developing human lung. In this study, we demonstrated that TRα1 mRNA expression increases from pseudoglandular to alveolar stage and then declines in adult specimens. Both TRα and TRβ proteins were expressed in nuclei of epithelial cells. TRβ was also present in endothelial cells of the arteries. The expression of TRα in mesenchymal cells combined with the expression of TRβ in endothelial cells is of interest in light of the observation that changes in the T₃ and T₄ levels have an effect on the heart and vascular system. Endothelium-dependent dilation of conductance vessels is impaired in hypothyroidism but augmented in hyperthyroidism. However, dilation of resistance vessels in skeletal muscle appears unchanged in both hypo- and hyperthyroidism. Treatment of rats with T₃ increased the endothelium-dependent...
relaxation of the renal artery, probably by increasing the vascular cAMP content.\textsuperscript{39} Particularly, the exposure to T\textsubscript{3} for 8 wk led to an enhanced expression of endothelial nitric oxide synthase and thus the release of NO, which appeared to be the predominant endothelium-derived vasodilator. In another study, Heron \textit{et al}.\textsuperscript{40} showed that 12 d of exposure to T\textsubscript{3} in neonates resulted in a considerable proliferation of coronary capillaries, which declined after 28 d of treatment. The expression of the two receptors in the endothelium and surrounding tissue during gestation may contribute to the expansion of the pulmonary vasculature, which is already present early in development.\textsuperscript{41}

RA is an oxidative metabolite of vitamin A and is involved in the control of many biological processes. The regulation of the differentiation processes by RA involves the ability of these signaling molecules to alter the expression of a wide variety of genes. For example, RA regulates the expression of \textit{hox} genes during embryonic branching morphogenesis to favor growth of proximal airways and to suppress distal epithelial bud formation.\textsuperscript{42,43}

RARs serve distinct functions in lung development. RXRs can act as homodimers or heterodimers with a variety of nuclear receptors, including RARs and TRs.\textsuperscript{43,44} RAR\textsubscript{α} mediates alveolar growth during the perinatal period of alveolarization.\textsuperscript{45} RAR\textsubscript{β} inhibits septation,\textsuperscript{46} whereas RAR\textsubscript{γ} is needed for normal lung elastin production and alveolarization.\textsuperscript{47} Administration of \textit{all-trans} RA to normal neonatal rats enhances alveolar septal formation without increasing alveolar surface area.\textsuperscript{11} RAR\textsubscript{α}/RXR\textsubscript{α} and RAR\textsubscript{α}/RXR\textsubscript{β} double-knockout mice were shown to have lung hypoplasia or agenesis, demonstrating the importance of these receptors during the early phases of lung development.\textsuperscript{13,14,48}

Previously, a study from Kimura \textit{et al}.\textsuperscript{28} has shown that the mRNA levels of RXR\textsubscript{γ} at proximal (trachea and main bronchus) and distal sites, of RAR\textsubscript{β} at distal sites, and of RAR\textsubscript{γ} at proximal sites were significantly higher in human fetal lung (13 - 16 wk gestation) compared with adults. In this study, we found no significant difference in mRNA expression between different developmental stages for the RARs and RXRs studied. However, we found positive correlations between gestation age and expression of RAR\textsubscript{α}, RXR\textsubscript{α}, and RXR\textsubscript{γ} from 13.5 - 41 wk.

In conclusion, mRNA expression of GR\textsubscript{α}, TRs, RARs, and RXRs were detected in human lung from 13.5 wk gestation onward. These results indicate that, as far as receptors are concerned, human fetal lung has the potential to respond to glucocorticoids, thyroid hormone, and RA as early as 13.5 wk gestation and potentially earlier, which implies an early potential for therapeutic benefits by exogenous analogs. However, more investigation is needed because there is a possibility of toxic effects by excess of endogenous ligands as well. We consider the results from this study as baseline data for further comparative studies involving abnormal lung development, such as in cases of pulmonary hypoplasia with or without diaphragmatic hernia.
ACKNOWLEDGEMENTS

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Spatial and Temporal Expression of Glucocorticoid, Retinoid, and Thyroid Hormone Receptors is not Altered in Lungs of Congenital Diaphragmatic Hernia


Pediatric Research 60(6):693-698, 2006
ABSTRACT
The degree of associated pulmonary hypoplasia and persistent pulmonary hypertension are major determination factors for survival in congenital diaphragmatic hernia (CDH) patients. Glucocorticoids, thyroid hormone, and vitamin A have been shown to be involved in human lung development. To determine their therapeutic potential in hypoplastic lungs of CDH patients, the temporal and spatial expression of glucocorticoid receptor, thyroid hormone receptors, retinoic acid receptors, and retinoid X receptors were evaluated in lungs of CDH patients, hypoplastic lungs from other causes, and normal lungs. As a series of supportive experiments, the expressions of these receptors were analyzed in lungs of nitrofen-induced CDH rats. Immunohistochemistry (human and rat) and in situ hybridization (rat) demonstrated no overt difference between CDH, hypoplastic, and control lungs, either in the localization nor the timing of the first expression of all analyzed receptors. The mRNA expression of each receptor was detected in all human CDH lungs by quantitative PCR. Our results suggest that, as far as receptors are concerned, hypoplastic lungs of fetuses and newborns with CDH are potentially as responsive to glucocorticoids, thyroid hormone, and retinoic acid as the lungs of normal children.
INTRODUCTION

CDH occurs approximately 1 in 2500 live births. This malformation, first described in 1848 by Vincent Bochdalek, is characterized by a diaphragmatic defect, herniation of abdominal viscera into the thoracic cavity, a variable extent of PH, and PPH. Despite the recent progress in new therapeutic strategies such as inhaled nitric oxide, exogenous surfactant therapy, or extracorporeal membrane oxygenation in selected infants, newborns with CDH continue to experience a high mortality. The major factors limiting survival of CDH patients are respiratory insufficiency secondary to PH and PPH. The lungs of newborns with CDH are characterized by a variable amount of immature alveoli with thickened walls and increased interstitial tissue, whereas surfactant deficiency may occur mainly due to surfactant inactivation instead of a primary surfactant deficiency.

With the increased use of prenatal ultrasound, more CDH cases are being diagnosed prenatally. Failure of postnatal therapies to significantly improve the prognosis of these infants has led to new strategies to rescue or stimulate fetal lung growth before delivery. For over two decades, pioneer efforts have been made to repair the diaphragmatic defect prenatally in fetuses with CDH but the outcome has been hampered by technical difficulties and a high rate of preterm delivery. The observation that infants with laryngeal atresia develop hyperplastic lungs led to the concept of "PLUG" (plug the lung until it grows) and fetal endoscopic temporary tracheal occlusion (FETENDO). A randomized clinical trial sponsored by the National Institutes of Health demonstrated no survival benefit compared with elective delivery in the specialist centers with optimal postnatal care. However, the trial continues in Europe with fetoscopic tracheal occlusion (FETO), hoping that further technical refinement may improve survival in high-risk patients.

Inasmuch as the benefit of fetal surgical interventions remains unclear, pharmacological approaches to stimulate prenatal lung growth and maturation have been considered as another strategy to improve lung development in case of CDH. GC are used worldwide to accelerate pulmonary development in threatened premature labor to decrease the incidence of respiratory distress syndrome. Within this context, the potential beneficial effects of prenatal GC treatment on lung maturation have been studied in the nitrofen (2, 4-dichlorophenyl- p-nitrophenyl ether)-induced CDH rat model and the surgical sheep model for CDH. The nitrofen model, based on the teratogenic effects of the herbicide, lead to a condition in rodents very similar to that observed in human CDH, including the features of PH and PPH. A disturbance in maternal thyroid function as well as in fetal thyroid function has been suggested to be involved in the teratogenic effect of nitrofen. Firstly, there is a stereo-chemical similarity between nitrofen and thyroid hormone. Secondly, nitrofen-treated dams have less malformed fetuses if T4 is given during pregnancy; and, thirdly, nitrofen-treated pregnant rats have lowered T4 and TSH levels. In addition, our group has demonstrated that nitrofen decreases the binding of T3 to the thyroid hormone receptor α1 and β1 in a noncompetitive way.
Recently, more attention has been drawn to vitamin A and its active metabolite, RA. The classical data published by Anderson\textsuperscript{14} showed that 25\% of rats born from dams with a vitamin A-deficient diet have a defect in the diaphragm. The incidence of a diaphragmatic defect reduced from 31\% to 24\% when a single dose of vitamin A administrated to vitamin A-deficient female rats on d 15 of pregnancy.\textsuperscript{15} Thebaud et al.\textsuperscript{16,17} have shown that antenatal treatment with vitamin A in the nitrofen-induced CDH model reduces the incidence of CDH at term as well as improving lung growth and maturation. In humans, lower plasma vitamin A levels were found in prematurely born infants, especially in patients with respiratory distress and in a selective number of term newborns with pulmonary hypoplasia associated with congenital diaphragmatic hernia.\textsuperscript{18,19} Although exciting, the confirmation of these findings awaits international collaboration, which is ongoing at present.

The actions of glucocorticoids, thyroid hormone, and retinoic acid are mediated through their specific nuclear receptors and the level of nuclear receptor expression determines cellular sensitivity to certain hormones.\textsuperscript{20} Since the optimal way of prenatal modulation of CDH is still a matter of ongoing debate, detailed information on the tissue distribution of these receptors in case of abnormal pulmonary development is warranted. Therefore, we examined the expression of GR\textsubscript{α}, TR, RAR, and RXR in human lungs with CDH, pulmonary hypoplasia due to other causes and compared these data with an ontogenic study of the same receptors during normal human lung development published by our group.\textsuperscript{21} As a series of supportive experiments, we examined the expression of these receptors in the lungs of nitrofen-induced CDH rats.

**MATERIALS AND METHODS**

**Human**

Following approval of the experimental design and protocols by the University Ethical Committee, lung tissues were retrieved from the archives of the Department of Pathology, Erasmus MC, Rotterdam. There were 18 CDH lung samples collected from either termination of pregnancy or patients who died within 48 h after birth (gestational age, 18 - 41 wk; mean, 34.2 wk). Demographic information of CDH cases is shown in Table 3.1. Immunohistochemical studies were performed in all CDH samples, whereas PCR was performed in five samples (cases 1 - 5) of which frozen tissue was available. Twenty paraffin-embedded lung samples from fetuses or newborns with pulmonary hypoplasia due to other causes, including Pena-Shokeir syndrome, hydrothorax, renal dysgenesis, and oligohydramnios (gestational age, 18 - 40 wk; mean, 30.5 wk) were included for immunohistochemical studies. None of the patients included in this study was subjected to prenatal steroid or extracorporeal membrane oxygenation therapy. Lung tissue from 15 age-matched fetuses and newborns (from termination of pregnancy or autopsies) without pulmonary abnormalities served as control (gestational age, 18 - 41 wk; mean, 30.4 wk). All samples were harvested within 24 h after death.
Table 3.1 Clinical information of congenital diaphragmatic hernia patients

<table>
<thead>
<tr>
<th>case</th>
<th>Gestational age (wk)</th>
<th>Birth weight (g)</th>
<th>Lung/body weight ratio*</th>
<th>Time of death after birth</th>
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<tr>
<td>1†</td>
<td>23</td>
<td>558</td>
<td>0.012</td>
<td>Termination of pregnancy</td>
</tr>
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<td>2†</td>
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<td>0.013</td>
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</tr>
<tr>
<td>3†</td>
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<td>2800</td>
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<tr>
<td>4†</td>
<td>38</td>
<td>2450</td>
<td>0.011</td>
<td>Minutes</td>
</tr>
<tr>
<td>5†</td>
<td>41</td>
<td>3510</td>
<td>0.006</td>
<td>48 h</td>
</tr>
<tr>
<td>6</td>
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<td>161</td>
<td>0.012</td>
<td>Termination of pregnancy</td>
</tr>
<tr>
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<td>21</td>
<td>416</td>
<td>0.007</td>
<td>Termination of pregnancy</td>
</tr>
<tr>
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<td>28</td>
<td>830</td>
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<td>2 h</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>1250</td>
<td>0.005</td>
<td>Minutes</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>2545</td>
<td>0.003</td>
<td>1 h</td>
</tr>
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<td>38</td>
<td>2870</td>
<td>0.005</td>
<td>30 min</td>
</tr>
<tr>
<td>12</td>
<td>38</td>
<td>2900</td>
<td>NA</td>
<td>24 h</td>
</tr>
<tr>
<td>13</td>
<td>39</td>
<td>2890</td>
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<td>15 min</td>
</tr>
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<td>40</td>
<td>1220</td>
<td>NA</td>
<td>24 h</td>
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<tr>
<td>16</td>
<td>40</td>
<td>2050</td>
<td>0.005</td>
<td>Minutes</td>
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<tr>
<td>17</td>
<td>41</td>
<td>2765</td>
<td>0.005</td>
<td>1.5 h</td>
</tr>
<tr>
<td>18</td>
<td>42</td>
<td>3355</td>
<td>0.002</td>
<td>30 min</td>
</tr>
</tbody>
</table>

* Normal lung/body weight ratio: before 25 wk of gestation = 0.015; from 25 wk of gestation = 0.012
† Frozen lung tissue available for quantitative PCR

Nitrofen-induced rats

Adult Wistar rats were obtained from the HSD animal farm in Zeist, the Netherlands. Animal experiments were performed in accordance with the guidelines of the animal research committee. The positive sperm plug day was designated as d 1 of gestation. To induce CDH and pulmonary hypoplasia, 100 mg of nitrofen dissolved in 1 ml olive oil was given to the dams by gavages on d 10. Fetuses were delivered by cesarean section at 15, 18, and 20 d of gestation, which corresponds to the pseudoglandular, the canalicular, and the saccular stage of rat pulmonary development respectively (term = 23 d). All tissues were fixed in 4% buffered formaldehyde and embedded in Paraplast Plus (Monoject, Kildare, Ireland). Seven-micrometer sections were cut and mounted onto RNase free 3-aminopropyltriethoxysilane coated slides (Sigma Chemical Co., St. Louis, MO). There were three to five random fetuses in each experimental group. From the group treated with nitrofen, only fetuses with a diaphragmatic hernia were included in this study.

Immunohistochemistry

Immunohistochemistry was performed on human and rat tissues for GRα, TRα, TRβ, RAR (αβγ), and RXR (αβγ) using a ChemMate DAKO EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (DakoCytomation B.V., Heverlee, Belgium). Primary antibodies and immunohistochemical processes were described in detail in our previous study. Negative controls were performed by omission of the primary antibodies. No
quantification of the staining intensity was attempted because the immunohistochemical study was basically to demonstrate the cellular distribution of the receptors.

**In situ hybridization**

Rat tissues were analyzed for GR, TRα, TRβ, RXRα, and RXRβ. GR cDNA (2.4 kb) was isolated from rat liver and recloned in pBluescript SK+ in the BamHI restriction site of the multiple cloning site (kind gift from Dr. Paul Godowski). Probes for the TRα and TRβ mRNA in EcoRI-HindIII cDNA fragments were obtained from H.C. Towle. RXRα and RXRβ mRNA were detected using an EcoRI-EcoRI cDNA fragment (1850 bp) and EcoRI-HindIII cDNA fragment (1360 bp), respectively. The hybridization conditions were as described elsewhere. The sections of rat fetuses were digested with 0.1% (wt/vol) pepsin (Sigma Chemical Co.) in 0.01 M HCl for 7 min (d 15 of gestation), 10 min (d 18 of gestation), and 15 min (d 20 of gestation) at 37 °C. The hybridization with [α-35S] dUTP labeled anti-sense probes was 16 - 18 h at 54 °C. The probe concentration was approximately 50 pg/µl and the specific activity of the anti-sense RNA probe was approximately 500 dpm/pg. After hybridization, the sections were washed and treated with RNase A. After exposure to photographic emulsion (Ilford Nuclear Research Emulsion G-5, Ilford, Cheshire, UK), the sections were counterstained with nuclear fast red, dehydrated in a graded series of ethanol and xylol, and mounted in Malinol (Chroma-Gesellschaft, Schmidt GmbH+Co, König, Germany).

**Real-time quantitative PCR**

Five CDH lungs and 15 control human lungs were analyzed for mRNA expression of GRα, TRα1, TRβ, RAR (α,β,γ), and RXR (α,β,γ). Total RNA was extracted from frozen human lung tissues using TRizol reagent (Invitrogen, Breda, the Netherlands), according to the manufacturer's instruction. Total RNA was quantified by measuring the absorbance at 260 nm and the purity was checked with 260/280 nm absorbance ratio. Reverse transcription and PCR conditions were carried out exactly as described before. PCR was run in triplicate for each sample. Negative control samples and reactions mixed without cDNA templates were run in parallel.

PCR results are shown as the relative expression level ($2^{-\Delta\Delta Ct}$) of normalized samples ($\Delta Ct$) in relation to the expression of the "calibrator" sample. The Ct value refers to the cycle number at which the PCR plot crosses the threshold line. $\Delta Ct$ is calculated by subtracting Ct value of the corresponding GAPDH control (endogenous reference control) from the specific Ct value of the target, and $\Delta\Delta Ct$ is obtained by subtracting the $\Delta Ct$ of each experimental sample from the $\Delta Ct$ of the "calibrator" sample. In this study, the control lung of 18 wk of gestation were used as a calibrator, which was arbitrarily set at 100% (arbitrary value = 1).
Figure 3.1 Relative mRNA expression of GRα, TRα1, TRβ, RARs (α,β,γ), and RXRs (α,β,γ) in human CDH and control lungs. All analyzed receptors were expressed in five lungs from CDH cases (●) and 15 control lungs (○). The expression levels are shown as relative value in an arbitrary unit compared to control lung of 18 wk of gestation (as outlined in "Materials and Methods").
**RESULTS**

**Human**

Five CDH lungs and 15 age-matched controls were analyzed with quantitative PCR. The mRNA expression for GRα, TRα1, TRβ, RAR (α,β,γ), and RXR (α,β,γ) was detected in all samples and the relative mRNA expression level of each gene is illustrated in Figure 3.1. No statistic analysis was done due to limited number of CDH samples. The lungs of 18 CDH cases and 20 patients with pulmonary hypoplasia secondary to causes other than CDH were included for immunohistochecmical studies. The expression of GRα, TRα, TRβ, RAR (α,β,γ), and RXR (α,β,γ) was detected in a similar fashion in all control, CDH, and pulmonary hypoplasia samples. GRα reactivity was detected mainly in epithelial cells (results not shown). TRα was expressed in both mesenchymal and epithelial cells (Figure 3.2, A-C), whereas TRβ reactivity was detected in the epithelial cells as well as in the endothelial cells of arteries (results not shown). RAR (α,β,γ) and RXR (α,β,γ) were expressed in virtually all epithelial and mesenchymal cells (Figure 3.2, F-H, for RXRγ, for others results not shown). The spatio-temporal expression patterns of all receptors were similar in the CDH lungs, pulmonary hypoplasia due to other causes, and normal lungs.

**Nitrofen-induced rat**

In a series of supportive experiments, the immunohistochemical studies demonstrated the expression of GRα, TRα, TRβ, RAR (α,β,γ), and RXR (α,β,γ) at the protein level in both control and nitrofen-induced CDH rat lungs. The staining pattern of each receptor was comparable to that observed in human lungs as shown in Figure 3.2 for TRα (Figure 3.2, D and E) and RXRγ (Figure 3.2, I and J), for others the results are data not shown. The results of the RNA *in situ* hybridization studies at d 15 and d 18 of gestation are shown in Figures 3.3 and 3.4, respectively. GR mRNA was observed in the endodermal part of the developing lung (d 15 of gestation) (Figure 3.3, A and B). On d 18 of gestation, GR expression was ubiquitous in both epithelium and mesenchyme, albeit more pronounced in the epithelium (Figure 3.4, A and B). The mRNA expression of RXRα and RXRβ was detected in both mesenchyme and epithelium from d 15 of gestation onward (Figure 3.3, C-F, and Figure 3.4, C-F), which corroborates the immunohistochemical findings. In contrast to the broad expression of TRα in both epithelial and mesenchymal cells as shown by the immunohistochemical studies (Figure 3.2, A-E), the expression of TRα mRNA in *in situ* hybridization studies was confined to the pulmonary mesenchyme (Figure 3.3, G and H, and Figure 3.4, G and H). The expression of TRβ mRNA was detected predominantly in the pulmonary epithelium of the distal conducting airways (Figure 3.3, I and J and Figure 3.4, I and J). Expression of TRβ mRNA was observed at all time points in pulmonary arteries, while expression of TRα mRNA was continuously observed in pulmonary veins. An accurate description of the expression patterns became more difficult near term (d 20 of gestation) because the walls of the airways become very thin due to expansion of the gas-exchange surface (results not shown). In nitrofen-induced rat lungs, the spatial and temporal expression pattern was similar to that observed in control lungs for all investigated receptors (Figures 3.3 and 3.4).
Figure 3.2  (for color figures see page 107) TRα and RXRγ expression pattern in human and rat lungs. TRα (A-E) and RXRγ (F-J) are seen in both epithelial and mesenchymal cells. There is no difference in the localization of TRα or RXRγ in the lungs of human control: 23 wk (A, F), CDH: 34 wk (B, G), or pulmonary hypoplasia due to other causes: 27 wk (C, H). Comparable patterns are observed in control (D, I) and nitrofen-induced CDH (E, J) rat lungs (d 18 of gestation). All pictures were taken at the same magnification; scale bar = 100 µm.
Figure 3.3 In situ hybridization studies of hormone receptors expression in normal (A, C, E, G) and nitrofen-induced CDH rat lungs (B, D, F, H) at d 15 of gestation. GR mRNA is observed in the endoderm lining the branching airways (A, B). RXRα mRNA (C, D) and RXRβ mRNA (E, F) are observed in both germ layers. TRα mRNA (G, H) is exclusively expressed in the mesenchyme in contrast to TRβ mRNA (I, J), which is expressed in the epithelium. All pictures were taken at the same magnification; scale bar = 500 µm.
Figure 3.4 In situ hybridization studies of hormone receptor expression in normal (A, C, E, G) and nitrofen-induced CDH rat lungs (B, D, F, H) at d 18 of gestation. At this stage, GR mRNA is faintly expressed in the entire lung (A, B). RXRα mRNA (C, D) and RXRβ mRNA (E, F) are expressed in both mesenchyme and epithelium. TRα mRNA (G, H) is expressed in the mesenchyme, while TR beta mRNA (I, J) is exclusively expressed in the epithelium. All pictures were taken at the same magnification; scale bar = 500 µm.
DISCUSSION

In this present study, no obvious differences in the spatial and temporal expression pattern of GRα, TR, RAR (α,β,γ), and RXR (α,β,γ) were observed between human CDH lungs, pulmonary hypoplasia from other causes and control lungs. These results are supported by a series of experiments undertaken in the nitrofen rat model for CDH. This study was inspired by the promising results obtained by the administration of glucocorticoids, thyroid hormone or vitamin A to nitrofen-treated rats. In our previous study, we have shown the expression of the nuclear hormone receptors in human lung from 13.5 wk until term with no change in localization throughout development. However, there is still limited information about the presence of these receptors in both human and rat CDH lungs. Antenatal corticosteroids are recommended by National Institutes of Health consensus to all fetuses between 24 and 34 wk of gestation at risk of preterm delivery. This will lead to an induction of surfactant production, an acceleration of lung maturation and an increase in lung compliance. In the present study, we demonstrate that the GRα is indeed expressed in a similar fashion in both normal lung tissues as well as in hypoplastic lungs of CDH cases or pulmonary hypoplasia due to other causes. The same holds true for control rat lungs and nitrofen-induced CDH lungs. At mRNA level, using quantitative PCR, we found no significant difference in GRα expression between CDH and control lungs. These findings are suggestive for a potential similar responsiveness to GC in lungs of CDH fetuses. There was one report of three CDH patients that suggested the benefit of prenatal GC for fetuses with CDH. But recently, the randomized trial and cohort study by the CDH study group demonstrated no significant benefit of late prenatal GC (after 34 wk of gestation) to fetuses with CDH, although more cases have to be included. With the paucity of human data, the side effects of GC and the lack of information on dosage and timing, antenatal GC are not recommended beyond 34 wk of pregnancy.

The role of thyroid hormone in lung development is exerted predominantly during the later phases of lung development, in which the regulation of surfactant production takes place. In rats, administration of T3 accelerates the process of septation, resulting in a greater alveolar surface area. Our study demonstrates that the localization of TR is not changed in hypoplastic lungs of fetuses with nitrofen-induced CDH. These results are compatible with the results from previous study by Tovar et al., which demonstrated that nitrofen leads to decreased levels of T3 and T4 in the plasma but not at the tissue level. This is an indication that induction of pulmonary hypoplasia in nitrofen-induced CDH is not the result of an alteration in the localization of TR. In contrast to this, we observed lower mRNA expression of TRα1 in human CDH lungs than in control lungs, which was in agreement with a previous study in nitrofen-induced CDH rats. Although the latter data are suggestive for a diminished TRα1 expression, we are aware that far-reaching conclusions cannot be drawn due to the limitations of our study, in particular the limited number of CDH lung samples available for PCR analysis.
Vitamin A is important for lung development. All-trans-RA acts via RAR, whereas 9-cis-RA exerts its effects via both RAR and RXR. RXR can act as homodimers or heterodimers with a variety of nuclear receptors, including RAR and TR.35,36 In animal studies, the lack or the excess of vitamin A during embryonic development results in congenital malformations such as infertility, anophthalmia, and lung hypoplasia.18,37,38 Transgenic RARα/RXRα and RARαβ double knockout mice were shown to have lung hypoplasia or agenesis.37-39 Our collaborative studies in the nitrofen rat model for CDH demonstrated that prenatal administration of vitamin A reduces the incidence of CDH and increases the levels of surfactant protein A and C in the offspring.16,17 In human pneumocytes cultured experiments, vitamin A administered after nitrofen exposure significantly increased the expression of surfactant protein B.40 In our present study, we showed that the expression pattern for all RAR and RXR is not changed in hypoplastic lungs of nitrofen-induced CDH rats. Similar findings are shown in human tissues for all RAR and RXR by immunohistochemical studies. Owing to the limited number of CDH samples, no statistical analysis was done. Although we observed a low RXRγ mRNA expression in human CDH lungs, there is possibly a redundancy for most functions of RXRγ by the other receptors of the family. The studies in RAR/RXR mutant mice revealed that there is a high degree of functional redundancy among these receptors.36

Our findings suggest that there is a potential for biologic effects of exogenous glucocorticoids, thyroid hormone, and retinoic acid in CDH lungs. Experimental studies have illustrated the potential benefits of antenatal corticosteroids and vitamin A to overcome pulmonary hypoplasia in animal models of CDH. Our results indicate that there might be a potential for similar effects in cases of human CDH patients as well. For instance, the current evaluation of vitamin A levels in newborn babies with CDH compared with age-matched controls47 and the experimental data obtained by Greer et al.41 potentially guide the use of prenatal modulation of pulmonary growth in human CDH in the near future. Very recently, a prospective international observational study on vitamin A levels in cord blood obtained institutional review board approval in Canada (Greer JJ, personal communication). Further advancement in therapies for CDH patients may arise from improved understanding of the mechanism of abnormal lung development.42

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Expression of Hypoxia-Inducible Factors in Normal Human Lung Development

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**ABSTRACT**

Pulmonary vascular development is essential for proper lung development and its disturbance can lead to neonatal morbidity and mortality, as exemplified in congenital diaphragmatic hernia. Hypoxia-inducible factors (HIFs) appear to be key molecules in physiologic and some forms of pathologic angiogenesis. Little is known about the qualitative and quantitative expression of HIFs in normal human fetal lung development. Therefore, we investigated the expressions of HIF-1α, HIF-2α and HIF-3α along with their upstream regulators and downstream targets, von Hippel-Lindau protein (pVHL), vascular endothelial growth factor A (VEGF-A) and its receptor, VEGFR-2, in 20 normal human fetal lungs (13.5 wk gestation till term) and five adult lungs. Quantitative PCR demonstrated a positive correlation between HIF-2α and VEGF-A expressions and gestational age. Although there appeared to be a decreasing trend in HIF-3α expression during pregnancy, it did not reach statistical significance. Immunohistochemistry for HIF-1α and HIF-2α revealed that while HIF-1α is expressed in the epithelium only, HIF-2α is expressed in both interstitium and epithelium. Our data suggest that hypoxia-inducible factors, most notably HIF-2α, appear to exert an important role in angiogenesis during human fetal lung development especially in the last phases of pregnancy, preparing the fetus for extra-uterine life. This action may occur through paracrine regulation between interstitial and epithelial cells, through an effect on downstream target genes, such as VEGF-A. As such our results form the baseline data for the evaluation and interpretation of abnormal pulmonary vascular development.
**INTRODUCTION**

Fetal lung development is an intricate process orchestrated by numerous growth and transcription factors, as well as morphogens. Structurally, the airway branches and pulmonary vessels are closely aligned, suggesting an intimate interaction during lung development. A vascular network has been shown to be present from the earliest stage of lung development and pulmonary vascular development seems to be rate-limiting for airway branching. The process of vascular development, in particular angiogenesis, is mainly driven by hypoxia. Moreover, the low-oxygen environment during fetal life is essential for normal development of the lung.

Vascular endothelial growth factor A (VEGF-A) is a potent hypoxia-inducible growth factor for vascular patterning during lung development. For instance, VEGF blockage impaired alveolar development and decreased pulmonary angiogenesis in newborn rats, mimicking bronchopulmonary dysplasia. Conversely, postnatal VEGF gene therapy improved survival and increased lung capillaries and alveolarization in this rat model. VEGF-A binds and activates two tyrosine kinase receptors, namely VEGFR-1, also known as fms-like-tyrosine kinase (Flt)-1, and VEGFR-2, also known as KDR in humans, or fetal liver kinase (Flk)-1 in mice. VEGFR-2 null mutant mice die in utero due to lack of vasculogenesis and defective in development of endothelial cells, indicative of the key role of VEGFR-2 in differentiation and/or proliferation of endothelial cells and vascular biology.

One of the most potent activators of VEGF transcription is hypoxia-inducible factor (HIF), which is a heterodimer composed of one of three oxygen-sensitive alpha subunits (HIF-1α, HIF-2α, HIF-3α) and a constitutive nuclear protein, the beta subunit (HIF-1β/ARNT). The alpha subunit is rapidly hydroxylated by prolyl hydroxylases (PHDs) under normoxic conditions, leading to its association with the von Hippel-Lindau protein (pVHL). Subsequently, the hydroxylated alpha subunit is targeted for proteasomal degradation. Since the PHDs require oxygen for their activity, the crucial hydroxylation step does not occur under hypoxic conditions and the alpha subunit remains stable and dimerizes with the beta subunit to form an active HIF transcription factor. Modifications to the HIF pathway have underlined its importance in development. Embryos with inactivating mutations of HIF-1α or HIF-2α all die in mid or late gestation.

Despite these studies in mice, very little is known about the VHL-HIF-VEGF pathway expression in human lung development. Although differential expression of the proteins HIF-1α and VHL has been observed in the lungs of CDH patients, quantitative analysis of HIFs during normal and abnormal lung development is limited. We therefore investigated the mRNA expressions of VHL, HIF-1α, HIF-2α, HIF-3α, VEGF-A and its receptor, VEGFR-2, by quantitative RT-PCR. In addition, we studied the expressions of HIF-1α and HIF-2α by immunohistochemistry in normal human fetal lungs, to determine their spatial expression in normal lung development.
MATERIAL AND METHODS

Tissue specimens
Normal human lung tissue (n = 25) was retrieved from the tissue bank of the Department of Pathology, Erasmus MC, Rotterdam, following approval of the experimental design and protocols by the Erasmus MC Medical Ethical Review Board Committee. Fetal and neonatal lungs (n = 20) were obtained from terminations of pregnancy and newborns who had died within 1 hour of birth. None of these fetuses or newborns had abnormalities related to the lungs and none of the newborns had received supplemental oxygen or mechanical ventilation. All tissues had been harvested within 24 hours after death. The lung samples were divided into four groups (n = 5) according to the lung developmental stages – i.e. pseudoglandular (7 - 17 wk gestation), canalicular (18 - 26 wk gestation), saccular (27 - 35 wk gestation), and alveolar (36 wk gestation – term). As a fifth group, unaffected lung adjacent to surgical resections for lung carcinoma was obtained from 5 adults (28 - 49 yr). Tissue specimens were snap-frozen, stored in liquid nitrogen until used for RNA isolation. For immunohistochemical studies, tissues were fixed by immersion in 4% buffered formalin and embedded in paraffin. Five-µm-thick paraffin sections were mounted on 3-amino-propyl-trioxysilane coated slides (Sigma, St. Louis, MO) and processed for immunohistochemistry.

RNA isolation and quantitative RT-PCR
Total RNA was extracted from frozen lung tissues using Trizol reagent (Life Technologies, Rockville, MD) following the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm and the purity was checked by the 260/280 nm absorbance ratio. cDNA synthesis was carried out in a final volume of 20 µl, containing 1 µg of total RNA, 50 mM Tris-HCL (pH 8.3), 75 mM KCl, 10 mM MgCl₂, 100 ng random hexamer primers, 0.5 mM dNTPs (Roche Diagnostics, Basel, Switzerland), 10 U RNase inhibitor, and 200 U Moloney Murine Leukaemia Virus reverse transcriptase (all reagents from Invitrogen, San Diego, CA). These samples were incubated for 1 hr at 37 °C followed by incubation for 15 min at 99 °C. Negative controls were prepared by omission of the Moloney Murine Leukaemia Virus enzyme.

Real-time PCR was performed using an iCycler IQ Real time PCR detection system (Bio-Rad, Veenendaal, The Netherlands) and qPCR Core kit for SYBR Green I (Eurogentech, Seraing, Belgium). Gene-specific primers used in this study are shown in Table 4.1. Gene amplification was carried out by activation of Hot Goldstar enzyme at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 sec, 58 °C for 30 sec, 60 °C for 45 sec, and 75 °C for 15 sec. Each sample was run in triplicate and negative control samples and samples without cDNA templates were run in parallel. After each assay, products were run on a 1.5% agarose gel to check the size and specificity.
Table 4.1 Primer sequences for quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5' → 3')</th>
<th>Reverse Primer (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>GCT CAT CAG TTG CCA TCT CC</td>
<td>CCT CAT GGT CAC ATG GAT GAG</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>CCA ATC CAG CAC CCA TCC CAC</td>
<td>GTT GTA GAT GAC CGT CCC CTG</td>
</tr>
<tr>
<td>HIF-3α</td>
<td>ACC TGG AAG GTG ACG TGG ACA CG</td>
<td>AAT CCT GTC GTG ACA GGA GG</td>
</tr>
<tr>
<td>VHL</td>
<td>CTC TCA ATG TTG ACG GAC AG CCA GAT CTT CGT AGA GC</td>
<td>CCA GAT CTT CGT AGA GC</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>AGA ATC ATC ACG AAG TGG TG</td>
<td>TGT TGT GCT GTA GGA AGC TC</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>CAG AGT GGC AGT GAG CAA AG CAC ACT CCA TGT TGG TC</td>
<td>TAC AGC ACT CCA TGT TGG TC</td>
</tr>
<tr>
<td>POLR2A</td>
<td>CGG ATG AAC TGA AGC GAA TG AGC AGA AGA AGA CAC AG</td>
<td>AGC AGA AGA AGA AGA CAC AG</td>
</tr>
</tbody>
</table>

PCR results are shown as the relative expression level of normalized samples (ΔCt) in relation to the expression of the ‘calibrator’ sample of 13.5 wk gestation (2^ΔCt), which was arbitrarily set at 100% (arbitrary value = 1). The Ct value refers to the cycle number at which the PCR plot crosses the threshold line; ΔCt is calculated by subtracting the Ct value of the corresponding endogenous reference gene; RNA Polymerase II, Subunit A (POLR2A) from the specific Ct value of the target gene, and ΔΔCt is obtained by subtracting the ΔCt of each experimental sample from the ΔCt of the ‘calibrator’ sample.

Statistical analysis
Data from real-time RT-PCR are shown as mean 2^ΔΔCt ± SEM. Differences in mRNA expression between groups were analyzed with one-way ANOVA with the post hoc least-significant difference (LSD) test. The correlations between mRNA expression and gestational age were determined by nonparametric Spearman's correlation. P ≤ 0.05 was considered statistically significant. All statistics were calculated using SPSS (version 11.0; SPSS Inc., Chicago, IL).

Immunohistochemistry
Antibodies used were mouse monoclonal anti-human HIF-1α H1o67 (Neomarkers, Fremont, CA) 1:1000, and rabbit polyclonal anti-mouse HIF-2α PM8 antiserum20 1:10,000. Formalin fixed paraffin embedded sections were dewaxed in two sequential xylene baths, and rehydrated using graded ethanol washes. For antigen retrieval, sections were immersed in preheated DAKO target retrieval solution (DAKO, Carpinteria, CA) and treated for 90 seconds in a pressure cooker. Incubation time with primary antibodies was 1 hour at room temperature. Antigen/antibody complexes were revealed by means of the Catalyzed Signal Amplification system (DAKO) according to the manufacturer's instructions. Sections were counterstained with haematoxylin for 15 seconds, dehydrated in graded ethanol washes, and mounted in DPX (Lamb, Eastbourne, United Kingdom).
RESULTS

Temporal expression (quantitative RT-PCR)
All investigated factors were expressed from 13.5 weeks gestation onwards to term, and in adults. In figure 4.1, the relative mRNA expression of each of the 6 factors investigated is depicted for each of the developmental stages and in the adult lung. Although there are reciprocal trends in the expression of HIF-2α and HIF-3α, with the former showing an increase throughout pregnancy and the latter showing a decrease, this does not reach statistical significance. However, there is a clear increase in the expression of VEGF-A near the end of pregnancy, i.e. in the alveolar phase, which is also apparent in adult lungs (P < 0.01 for alveolar or adult phase vs. pseudoglandular, canalicular, or saccular phase). No obvious increase or decrease in the expression of VHL, HIF-1α, or VEGFR-2 can be seen.

When the expression levels of all individual fetal samples are plotted against gestational age, HIF-2α and VEGF-A show a positive correlation in the course of fetal lung development (HIF-2α: r = 0.249, P = 0.023; VEGF-A: r = 0.587, P < 0.01) (Figure 4.2).

Figure 4.1 mRNA expression of VHL, VEGF-A, VEGFR-2, HIF-1α, HIF-2α, and HIF3-α in human lungs. Samples were grouped according to their developmental stages i.e. Pseudoglandular (Pseu), Canalicular (Can), Saccular (Sac), Alveolar (Alv) and Adult (Ad); n = 5 per group. Data are shown as mean 2^ΔΔCt ± SEM (as outlined in Materials and Methods). VEGF-A expression is significantly higher in the alveolar stage and in adult (*, P < 0.01). There was no significant difference in mRNA expression in other genes (P > 0.05).
Figure 4.2 Positive correlation between HIF-2α and VEGF-A mRNA expression and gestational age of 20 samples from 13.5 to 41 weeks of gestation. The r-values show Spearman’s correlation coefficient with gestational age.

Immunohistochemistry for HIF-1α and HIF-2α
The immunostaining for HIF-1α and HIF-2α revealed that both subunits were expressed in all tissues examined. HIF-1α immunoreactivity was primarily detected in the airway epithelium and showed a consistent pattern throughout gestation (Figure 4.3a-d). HIF-2α reactivity was detected mainly in the interstitium at early stages of lung development (Figure 4.4a, b), whereas its expression in the airway epithelium, predominantly in type II pneumocytes, was detected later on the gestation (Figure 4.4c, d).

DISCUSSION
In this study, we analyzed the expression of factors involved in the VHL-HIF-VEGF pathway during human lung development. Our quantitative PCR data demonstrated that VHL, HIF-1α, HIF-2α, HIF-3α, VEGF-A and VEGFR-2 are expressed in human lung tissue from 13.5 weeks of gestation onwards. Moreover, HIF-2α and VEGF-A mRNA expressions increase with advancing gestation. Immunohistochemistry was performed to show sites of HIF-1α and HIF-2α expression. HIF-1α expression was consistently detected in the airway epithelium throughout gestation. HIF-2α expression was first detected in the interstitium, while later on in gestation its expression was detected in type II pneumocytes. The sites of expression of VHL and VEGF were shown in our previous work.19
Chapter 4

Figure 4.3 (for color figures see page 108) HIF-1α immunostaining with strong expression in airway epithelium of human fetal lung at different gestational ages; (a) 16 wk, (b) 21 wk, (c) 27 wk, and (d) 31 wk. (Magnification, X 400)

Figure 4.4 (for color figures see page 109) HIF-2α immunostaining with predominant reactivity in the interstitium at early stages of human fetal lung development; (a) 16 wk, (b) 21 wk. Later on in gestation, HIF-2α expression was also detected in type II pneumocytes; (c) 27 wk, and (d) 33 wk. (Magnification, X 400)
The intimate relationship between the pulmonary vasculature and the airways suggests that these two systems interact during development. Recent reports have indeed indicated an essential role for vascularization in airway development. In utero pulmonary development occurs in a relatively hypoxic environment and one of the most potent angiogenic factors, VEGF-A, is actively transcribed under hypoxic conditions. VEGF-A transcription is upregulated in a hypoxic environment by one of oxygen sensitive HIF subunits. Our PCR data showed a positive correlation between gestational age and mRNA expressions of HIF-2α and VEGF-A. The mRNA expressions of HIF-1α and HIF-3α do not significantly change during fetal lung development. These results are compatible with previous studies in mouse lungs that showed increased expressions of HIF-2α and VEGF during development, whereas HIF-1α expression remained constant. Animal studies have shown that HIF-2α deficiency and the lack of VEGF in later stages of lung development result in impaired lung maturation and insufficient surfactant production. Using immunohistochemistry, we demonstrated that HIF-1α is mainly expressed in the bronchial epithelium throughout gestation, whereas HIF-2α is predominantly expressed in the interstitium in earlier stages and appears in the epithelium later in development. The expression of HIF-2α in the airway epithelial cells is in agreement with previous reports demonstrating HIF-2α expression in type II pneumocytes in mouse lungs. The difference in the expression patterns between HIF-1α and HIF-2α may reflect their individual roles in lung development.

HIF-1α and HIF-2α share a high degree of structural and functional similarity as emphasized by their ability to interact with hypoxia response elements of the target genes and the subsequent induction of transcriptional activity. However, ample evidence suggests that these two alpha subunits also have distinct physiologic roles. While HIF-1α is ubiquitously expressed, HIF-2α transcripts are more restricted to particular cell types, for instance vascular endothelial cells, type II pneumocytes and liver parenchyma. Analysis in embryonic stem cells suggested that HIF-1α, but not HIF-2α, plays an important role in hypoxia responses. In contrast to the stem cells studies, HIF-2α has been shown to be crucial in stimulating several hypoxia-inducible genes (including tyrosine hydroxylase and VEGF) during embryonic development. Moreover, a growing number of differences between HIF-1α and HIF-2α with respect to cell-specific regulation, hypoxic and non-hypoxic stimuli, interaction partners, and downstream targets is being reported. In animal studies, HIF-1α−/− mice exhibit midgestational lethality with severe blood vessel defects. Although homozygous HIF-2α knockout embryos also showed a defect in vascular remodeling, with aberrant vascular formations, a subset of the offspring survived to term but suffer from respiratory distress due to surfactant insufficiency.

A third HIF protein, HIF-3α, is also able to dimerize with HIF-1β and binds to hypoxia response elements. Among several splice variants of HIF-3α, inhibitory PAS domain protein (IPAS) is the best characterized. In adult mice under normoxic conditions, IPAS is predominantly expressed in Purkinje cells and corneal epithelium. However, IPAS can
also be induced by hypoxia in the heart and lung. In contrast to the role of HIF-1α and HIF-2α, IPAS has no endogenous transactivation function and has been reported to act as a dominant negative regulator of HIF-1α.24,32

The VEGF signaling pathway has been shown to play a crucial role in embryonic vasculogenesis, particularly during fetal lung development.8,10-12 The level of VEGF is critical for normal lung development. Overexpression of VEGF in distal lung altered vascularization and arrested airway branching, whereas a decrease in lung VEGF resulted in poor septal formation and an emphysematous pattern.33 In addition, reduced VEGF levels in tracheal aspirates were also observed in infants with bronchopulmonary dysplasia.34,35

It has been shown that the VEGF-pathway is important for both vascular development and branching morphogenesis.5,6,36 The roles of VEGF-A and its receptors in lung development were anticipated by their expression patterns. VEGF–A is expressed mainly in the epithelial cells, whereas VEGFRs are expressed in the mesenchymal cells. VEGF-A is also detected in alveolar type 2 cells and has an effect on the synthesis of surfactant protein (SP). VEGF-treated human lung explants demonstrated increased SP-A and SP-C mRNA compared with control lungs (38). Furthermore, VEGF could rescue HIF-2α+/− mice, which showed defective surfactant production, alveolar septal vascular defects and respiratory distress at birth.18 Our finding of increasing VEGF-A mRNA expression in fetal human lung during development suggests that the role of VEGF-A is not restricted to the initial phase of pulmonary development. Previous studies in VEGF-A knock-out mouse embryos have shown that VEGF-A is also necessary for events later in gestation such as vessel sprouting and maintenance of vessel integrity.19,40

It appears that VEGFR-2 is the main receptor mediating the effect of VEGF-A on lung maturation. The study by Compernolle and colleagues in mice with antibodies against VEGFR-1 and VEGFR-2 indicates that only VEGFR-2 mediates the effects of endogenous VEGF on lung maturation in vivo.18 Thus far, there are limited data on the ontogeny of VEGFR-2 in human lung development. In this study, we demonstrated no significant change of VEGFR-2 mRNA expression during human fetal lung development.

Taken together, we have shown that hypoxia-inducible factors, particularly HIF-2α, appear to exert an important role in vascular growth and airway branching morphogenesis in human fetal lung, especially in the last phases of pregnancy. The suggestive anti-correlation between HIF-2α and HIF-3α indicates a putative important regulatory role. This action occurs by regulation from interstitial and epithelial cells through an effect on downstream target genes, such as VEGF-A. These data contribute to our basic knowledge about normal pulmonary (vascular) development and may serve as a reference for the interpretation of pathological states such as primary pulmonary hypertension and congenital diaphragmatic hernia.
REFERENCES


The Effect of Oxygen Concentration on Hypoxia-Inducible Factors Expression in Human Fetal Lung Explants

Prapapan Rajatapiti, Jessica D. de Rooij, Dick Tibboel, Ronald R. de Krijger, and Robbert Rottier
ABSTRACT

Many investigations suggest that fetal lung development requires proper coordination between lung epithelial and vascular morphogenesis. A major determinant in lung vascular development is vascular endothelial growth factor (VEGF), which is regulated by hypoxia-inducible factors (HIFs). While VEGF expression is limited to airway epithelium, its receptors are expressed in pulmonary mesenchyme. Relatively low oxygen environment in utero is beneficial for fetal organogenesis, especially vascular development. Herein, we used a fetal human lung explant culture model to investigate the effects of hypoxia on fetal lung morphology and the mRNA expressions of VEGF, VEGFR-2, HIF-2α, and HIF-3α. The morphology remained largely unchanged in explants cultured at hypoxia compared with explants cultured at normoxia, and cell specific markers confirmed the viability of the explants after culturing for 6 days. Quantitative PCR showed that the mRNA expression of VEGF-A, but not VEGFR-2 is upregulated in explants cultured at 1.5% oxygen compared with 20% oxygen. We observed slight increases in HIF-2α and HIF-3α mRNA expressions in explants cultured at 1.5% oxygen compared with 20% oxygen; however the differences are not significant. These data suggest that the expression of VEGF and possibly that of HIF-2α is regulated by hypoxia in the developing human lung. Human lung explant culture appears to be a valuable model to unravel the molecular mechanism of factors that are known to modulate human lung development such as steroid hormones and retinoic acid.
INTRODUCTION

The molecular basis of pulmonary development has been studied extensively over the past few decades. Many morphogens, growth and transcription factors have been shown to play key roles in different stages of fetal lung development.\textsuperscript{1-3} Pulmonary development is a sophisticated process requiring reciprocal interactions between epithelium and mesenchymal cells. Accumulating evidence underlines the importance of vascular development to distal airway growth and development.\textsuperscript{4-6} It is important to note that normal pulmonary development occurs in a relatively hypoxic environment. A number of studies have shown that the relatively low oxygen environment of the fetus is favorable for embryo development and organogenesis.\textsuperscript{5,7-9}

The cellular responses to oxygen alteration in most mammalian systems are mediated by hypoxia-inducible factors (HIFs), which are known to control more than 100 genes.\textsuperscript{10} The HIF transcriptional complex is a heterodimer composed of one of the three oxygen-sensitive alpha subunits (HIF-1\textalpha, HIF-2\textalpha, or HIF-3\textalpha) and the constitutive beta subunit (HIF-1\textbeta/ARNT).\textsuperscript{11,12} Under hypoxic conditions, the HIF heterodimer accumulates in the nucleus and binds to hypoxia response elements of target genes, thereby regulating their transcription. In normoxia, hydroxylation of HIF-\alpha subunit by prolyl hydroxylases (PHDs) mediates interactions with the von Hippel-Lindau protein (pVHL), which subsequently leads to its ubiquitination and targets HIF-\alpha for proteasomal destruction.\textsuperscript{11,13}

One of the most potent hypoxia-inducible growth factors is vascular endothelial growth factor-A (VEGF-A). VEGF-A signals through two high affinity tyrosine kinase receptors, VEGFR-1 (Flt-1), and VEGFR-2 (KDR in human or Flk-1 in mouse). In the lung, VEGF-A functions as a mitogen and differentiation factor for endothelial cells.\textsuperscript{14} While VEGF-A is expressed mainly in the epithelial cells of the lung,\textsuperscript{15} VEGFRs are expressed in the mesenchymal cells. The level of VEGF is critical for normal lung development. Overexpression of VEGF in distal lung alters vascularization and arrests airway branching,\textsuperscript{16} whereas inhibition of VEGF results in less complex alveolar patterning and immature lung formation.\textsuperscript{17} The evidence that homozygous null VEGFR-2 mice die \textit{in utero} as a result of a lack of mature endothelial cells and absence of blood island formation, suggests a role of VEGFR-2 in mediating the mitogenic and chemotactic effects of VEGF-A on the endothelial cells.\textsuperscript{18}

In vitro studies using rat lung explants cultured at 3\% oxygen showed an increase in epithelial branching and cellular proliferation.\textsuperscript{5} Previous studies in transgenic mouse lungs showed the increase of both epithelial and endothelial branching morphogenesis in explants cultured at 3\% oxygen compared with 20\% oxygen. Additionally, by using antisense oligonucleotides against HIF-1\textalpha and VEGF, our group previously demonstrated that epithelial branching morphogenesis is drastically abolished when pulmonary vascular development is inhibited.\textsuperscript{5}
Based on the aforementioned information, a low oxygen environment of the fetus seems critical for pulmonary angiogenesis and possibly airway branching morphogenesis. Human lung explants maintained in vitro were used in a number of studies on lung development. For instance, Acarregui et al. previously showed the effect of low oxygen and cAMP on VEGF mRNA expression. However, very little data are available regarding the expressions of HIF-2α, HIF-3α and VEGFR-2 in human fetal lung. In this study we therefore used human fetal lung explants maintained in vitro to study the influence of low oxygen tension on the expressions of HIF-2α, HIF-3α, VEGF-A, and VEGFR-2 in the developing human lung.

**MATERIALS AND METHODS**

**Lung tissues and explants culture**
Lung tissues were obtained from human abortuses of 16 to 22 weeks of gestation, following the approval of the Erasmus MC Ethical Review Board of the experimental design and protocols and informed consent from the pregnant women. Fetal lung tissue was carefully separated from major blood vessels and airways, and dissected into 1 to 2 mm³ pieces. Lung explants (2 - 3 pieces) were placed on Nucleopore membranes (pore size 8µm; Whatman, Den Bosch, Netherlands), and incubated as air-liquid interface cultures in serum-free Waymouth's MB752/1 medium (GIBCO, Breda, Netherlands) with 1% Penicillin-Streptomycin and 1% Insulin-Transferrin-Selenium (GIBCO). Tissues were maintained either under standard culture incubator conditions of 37°C and 5% CO₂/95% air (20% oxygen; normoxia), or in an incubator with 1.5% O₂, 5% CO₂, and 93.5% N₂ (hypoxia). The culture medium was changed every 24 hours and tissues were harvested after 3 or 6 days of culture in normoxia or hypoxia.

**Immunohistochemistry**
Immunohistochemistry was performed using a standard avidin-biotin complex method. In brief, after deparaffinization for 10 min in xylene and rehydration through graded alcohol, slides were treated with 3% H₂O₂ in methanol to block the endogenous peroxidase activity. For antigen retrieval, slides were subjected to microwave treatment in citric acid buffer, pH 6.0 (Ki-67, TTF-1) or pronase (CD-31). After the blocking step, slides were incubated for 30 min at room temperature with a primary antibody against Ki-67 (1:150, Dako, Heverlee, Belgium), TTF-1 (1:100, Ab-1, Neomarkers, CA, USA), or CD-31 (1:30, clone MIB-1, Dako). After rinsing with PBS, slides were incubated for 10 min with a biotinylated secondary antibody (Labvision, CA, USA), followed by 10 min incubation with peroxidase-conjugated streptavidin (Labvision). Peroxidase activity was detected by diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) with 0.3% H₂O₂ and counterstained with hematoxylin. Negative controls were replicated by omission of the primary antibodies.
RNA isolation and quantitative RT-PCR
RNA from uncultured lung tissue and explants that had been cultured for 3 and 6 days were used for quantitative PCR analysis. Total RNA was isolated using Trizol reagent (Invitrogen, Breda, Netherlands) according to the manufacturer’s instructions. RNA was quantified by measuring the absorbance at 260 nm and the purity was checked by the 260/280 nm absorbance ratio. Total RNA (1 µg) was added to a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 100 ng random hexamer primer, 500 µM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP and dTTP), 10 U RNAse inhibitor and 200 U Moloney Murine Leukaemia Virus Reverse transcriptase (all reagents were obtained from Invitrogen). The RT thermal cycle was 1 hour at 37 °C followed by incubation for 15 min at 99°C.

Real-time PCR was performed using an iCycler IQ Real time PCR detection system (Bio-Rad, Veenendaal, The Netherlands) and qPCR Core kit for SYBR Green I (Eurogentech, Seraing, Belgium) under the following conditions: 10 min of initial denaturation at 95°C followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, 60°C for 30 s, and 75°C for 15 s. The sequences for gene-specific primers used in this study are described previously in Chapter 4. To verify the specificity of the amplified products, each PCR was followed by a melting curve analysis from 55°C to 95°C. Each sample was run as a triplicate and mRNA of each target gene was determined simultaneously in a 96-wells plate. Negative (no enzyme) and no-template (no cDNA) controls were also included.

For the relative quantitation, PCR signals were normalized using RNA Polymerase II, Subunit A (POLR2A) as an internal reference. The fold change was calculated as \(2^{-\Delta\Delta C_{\text{t}}}}\) according to Livak and Schmittgen. Lung tissue from day 0 (starting material) was used as a control group (arbitrary value = 1).

Statistical analysis
Data from the quantitative PCR are presented as mean \(2^{-\Delta\Delta C_{\text{t}}}} \pm \text{SEM}. The differences between experimental groups were evaluated by using one-way ANOVA with post hoc least significant difference test. \(P < 0.05\) was considered statistically significant. All statistics were calculated using SPSS statistical package (version 11.0; SPSS Inc., Chicago, IL).

RESULTS
Morphology of human fetal lung explants cultured at 20% oxygen
To evaluate the feasibility of fetal lung explants maintained in vitro under our conditions, human fetal lung explants derived from mid-trimester abortuses were maintained at 20% oxygen for 6 days. The microscopic appearance of the explants after culturing for 3 and 6 days (Figure 5.1B and C respectively) resembled the pseudoglandular stage of lung development (Figure 5.1A); except that the airways were dilated and the epithelial cells appeared flattened.
Figure 5.1 (for color figures see page 110) Morphology of human fetal lung explants cultured at 20% oxygen. Hematoxylin & Eosin staining of uncultured lung (A) resembled the pseudoglandular stage of lung development. The airways were dilated after the explants were cultured for 3 (B) and 6 days (C) at 20% oxygen. Bar, 500 µm.

To evaluate cell differentiation characteristics of the explants, immunohistochemistry with markers for epithelial cells (TTF-1), endothelial cells (CD-31) and proliferation (Ki-67) was performed on sections of explants cultured for 3 and 6 days. There was no difference in expression patterns of all markers between uncultured lungs and explants cultured for 3 or 6 days at 20% oxygen. Virtually all epithelial cells are positive for TTF-1 staining (Figure 5.2A, D, G), whereas endothelial cells are positive for CD-31 (Figure 5.2B, E, H). Ki-67 immunoreactivity was observed in both epithelial and mesenchymal cells (Figure 5.2C, F, I). Double immunohistochemical staining with Ki-67 and CD-31 showed ongoing endothelial proliferation in the explants cultured at normoxia after 6 days in culture (Figure 5.3B).

Morphologic changes associated with hypoxic exposure of fetal lung explants
Lung sections were immunostained with Ki-67, TTF-1 and CD-31 after the explants had been cultured for 6 days at either normoxic or hypoxic conditions, and uncultured tissue was used as control. The airways were larger in both hypoxic and normoxic cultured lung at day 6 compared with control. There were no differences in the localization of TTF-1 (Figure 5.2G, J) and CD-31 (Figure 5.2H, K) between explants cultured at 20% and 1.5% oxygen. However, CD-31 immunoreactivity appeared to be stronger in hypoxic-exposed lung compared with explants cultured at normoxia (Figure 5.2K vs. 5.2H). A proliferation marker, Ki-67, was expressed in both epithelium and mesenchymal cells in control and explants cultured at 20% oxygen (Figure 5.2C, F, I) but appeared to be more restricted to the epithelium in explants cultured at 1.5% oxygen (Figure 5.2L).

Quantitative RT-PCR
Quantitative PCR showed that VEGF-A, VEGFR-2, HIF-2α, and HIF-3α mRNA were expressed in all experimental groups (n = 5 per group). The relative mRNA expression of each gene is shown as mean ± SEM in Figure 5.4. There was an increase in VEGF-A mRNA expression in explants cultured at 1.5% oxygen compared with explants cultured at 20% oxygen and control (P ≤ 0.01). However, under hypoxic conditions, VEGF-A mRNA expression was downregulated in explants cultured for 6 days compared with 3 days (P < 0.01). There was no significant change in the expression of VEGFR-2 between the explants cultured in normoxia and hypoxia. Although mRNA expression of HIF-2α
(day 3) and HIF-3α (day 6) was slightly higher in the explants cultured at 1.5% oxygen compared with explants cultured at 20% oxygen, the difference did not reach statistical significance.

Figure 5.2 (for color figures see page 111) Immunohistochemical staining with TTF-1, CD-31, and Ki-67 of uncultured lung (A–C), explants cultured at 20% for 3 (D–F) or 6 days (G–I), and 1.5% oxygen for 6 days (J–L). TTF-1 immunoreactivity is detected in epithelial cells (A, D, G, J). Explants cultured at 1.5% oxygen (K) showed stronger CD-31 staining in the endothelial cells compared with control (B) and explants cultured at 20% oxygen (E, H). Ki-67 staining (C, F, I, L) showed proliferating epithelial cells and mesenchymal cells in control (C) and explants kept at 20% oxygen (F, I), whereas the expression is more restricted to mesenchyme in explants kept at 1.5% oxygen (L). Bar, 500 µm.
Figure 5.3 (for color figures see page 112) Double immunostaining of Ki-67 and CD-31 on explants cultured at 20% oxygen for 6 days. Ki-67 (A) immunoreactivity is detected in epithelium and mesenchyme. Double staining with CD-31 (pink staining) shows proliferating endothelial cells (arrows; B). Bar, 250 µm.

Figure 5.4 Relative mRNA expression of VEGF-A, VEGFR-2, HIF-2α, and HIF-3α in human fetal lung explants maintained for 3 or 6 days in 20% oxygen (Nd3 and Nd6, respectively) or 1.5% oxygen (Hd3 and Hd6, respectively) and uncultured lungs (d0). Bar represent means ± SEM (n = 5). VEGF-A mRNA expression is upregulated in explants cultured at 1.5% oxygen (Hd3 and Hd6) compared with explants cultured at 20% oxygen (Nd3 and Nd6) and control (d0) but its expression in explants kept at hypoxic conditions declines when cultured for a longer period (Hd6 vs. Hd3). * P ≤ 0.01 vs. d0, Nd3, and Nd6. # P < 0.01 vs. Hd3.
DISCUSSION

The developing lung requires the formation and maintenance of a vascular network in close proximity to a layer of alveolar epithelial cells. In this study, we demonstrated that human lung explants cultured for 3 to 6 days at 1.5% oxygen maintain appropriate proliferation and differentiation of the airway epithelium as well as endothelial cells. Moreover, we demonstrated that VEGF-A mRNA expression was increased in the explants cultured under hypoxic conditions. There were no significant changes in the mRNA expressions of VEGFR-2, HIF-2α, and HIF-3α.

Maintaining oxygen homeostasis is essential for tissue development, growth, and preservation of structural integrity. Normal fetal development takes place in a relatively hypoxic environment and hypoxia is known to be an important regulator of angiogenesis. Fetal lung explants maintained in vitro are a well-characterized model for studying fetal lung development. The differentiation of midgestational human fetal lung explants in culture has been characterized both biochemically and morphologically as represented in table 5.1, which contains an overview of the difference studies published. For instance, the distal epithelium of human fetal lung tissues differentiates spontaneously into type II pneumocytes in an atmosphere of 20% oxygen. However, there is little information on the role of hypoxia at the cellular level in embryonic lung development. Previous studies in murine lungs have shown that hypoxia, relative to ambient oxygen condition, enhances the development of pulmonary vasculature and airway epithelial branching morphogenesis. In the present study we observed that the airway epithelium explants appeared flattened within the dilated airspace after being maintained at 1.5% oxygen for 6 days. The immunohistochemical studies with epithelium and endothelium specific markers showed no obvious differences in the localization of TTF-1 and CD-31 between human lung explants cultured at 1.5% oxygen or 20% oxygen. Ki-67, a proliferation marker, showed that cells in the lung explants maintain their character and are still dividing. A formal quantification of the proliferating cells was not performed.

The close anatomical relationship between airways and blood vessels in the lung suggested their putative interaction during development. The inhibition of vascularization in mouse lungs in vitro resulted in a decrease in epithelial branching morphogenesis. In addition, Schwarz et al. showed that inhibition of vascular development with endothelial monocyte activating polypeptide II (EMAPII) resulted in an inhibition of airway epithelial morphogenesis. Many studies have provided evidence that VEGF acts as a potent inducer of endothelial cell growth and that hypoxia is one of its important stimuli. VEGF is essential for embryonic development as it was shown that inactivation of a single VEGF allele results in embryonic lethality with impaired vessel formation. In this study, we showed that VEGF-A mRNA expression is significantly upregulated in lung explants cultured at 1.5% oxygen for 3 and 6 days as compared to explants cultured at normoxic conditions. Similar findings have been reported for VEGF expression in human, mouse, and rat fetal lung explants cultured at low oxygen. These results
confirm that low oxygen stimulates VEGF expression and possibly vascular development in lung explants.

While VEGF has been detected mainly in the distal epithelium of the fetal lung and its expression increases as the lung develops, its receptors are strongly expressed in the mesenchyme. vEGF and its receptors being expressed in adjacent tissues suggest a signaling loop between these two components during lung development. It has been shown that VEGF-A signaling through VEGFR-2 is important not only for vasculogenesis but also for triggering an increase in pulmonary epithelial morphogenesis. The VEGFR-2 blocker SU-5416 causes a decrease in capillary density and inhibits septation, leading to lung immaturity. VEGFR-2 expression has been shown to be stimulated by HIF-2α but no oxygen-dependent upregulation of VEGFR-2 expression in human lung has been reported. Previous studies in a transgenic mouse model showed that after two days' culture VEGFR-2 mRNA expression was increased in mouse lung explants cultured under hypoxic conditions compared to explants cultured under normoxic conditions. However, when the explants were cultured for a longer period, this significant difference was no longer noticeable. Our results showed no difference in VEGFR-2 mRNA expression in human lung explants cultured for 3 or 6 days at 1.5% oxygen compared with explants cultured at 20% oxygen. Together with findings from previous studies in mice, these results suggest that the effect of hypoxia on VEGFR-2 mRNA expression is transient.

VEGF gene transcription is activated by hypoxia through hypoxia-inducible transcription factors. HIF-1α and HIF-2α have a close sequence similarity but their modes of expression vary greatly. HIF-2α was reported to be abundantly expressed in murine adult lungs under normoxic conditions. Although HIF-2α-null mice exhibit embryonic lethality with abnormal lung maturation and blood vessel defects or a defect in catecholamine production, a subset of HIF-2α knockout offspring survived postnatally but suffered from respiratory distress due to surfactant deficiency. HIF-3α, which was discovered recently, appears to be involved in negative regulation of the angiogenic response through an alternative splice variant, inhibitory PAS domain protein (IPAS). IPAS can be induced by hypoxia in heart and lung resulting in a negative feedback loop for HIF-1α activity in these tissues. To date very little data are available regarding the expression and function of HIF-3α. A previous study in adult mice by Heidbreder et al. reported that mRNA expression of HIF-3α, but not HIF-2α increased significantly corresponding to the duration of systemic hypoxia. In addition, they found an increase in both HIF-2α and HIF-3α protein levels. Our quantitative PCR show a slightly increase in HIF-2α mRNA expression in explants cultured under hypoxic condition at day 3 but the difference is not significant. There is also no significant hypoxic induction of HIF-3α mRNA expression in human fetal lung explants. This can be explained by the fact that HIF expression is mainly regulated at the posttranslational level. However, in combination with our observation that VEGF-A, one of the prime targets for HIF-2α
transcription, is dramatically induced in the explants cultured at 1.5% oxygen, it appears that the hypoxic conditions induce HIF-2α expression as well.

In summary, this study has demonstrated that fetal human lung explants exposed to relative hypoxia (1.5% oxygen) maintain proper epithelial and mesenchymal morphogenesis. The increase in VEGF-A expression under hypoxic conditions suggests a role for VEGF-A in regulating pulmonary vascular and airway development. Human fetal lung explants maintained in a serum-free system allowed us to focus on the local effects of oxygen tension rather than on a systemic response to hypoxia. This model can be applied to investigate the effects of different ligands that influence lung development, such as retinoic acid and steroid hormone. Moreover, this reproducible model of human embryonic lung cultures makes it possible to culture lungs under conditions of abnormal lung development, such as pulmonary hypoplasia resulting from obstructive uropathy and oligohydramnios, or congenital diaphragmatic hernia.
### Table 5.1
Previous studies with human fetal lung explants

<table>
<thead>
<tr>
<th>Point of interest</th>
<th>Culture condition</th>
<th>Main results</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Morphological change</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cell differentiation</td>
<td>20% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Spontaneous differentiation of airway epithelium into type II pneumocytes</td>
<td>(20)</td>
</tr>
<tr>
<td>apoptosis</td>
<td>20% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↑ number of cells undergoing apoptosis esp. in the interstitium</td>
<td>(25)</td>
</tr>
<tr>
<td>in response to hyperoxia</td>
<td>95% vs. 20% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↓ number of vessels and VEGF mRNA expression in hyperoxia</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ cell proliferation in the interstitium but not in epithelium</td>
<td></td>
</tr>
<tr>
<td><strong>Surfactant protein</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>effects of O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>70% or 95% vs. 20%</td>
<td>↑ SP-A, SP-C mRNA, SP-B unchanged</td>
<td>(23, 24)</td>
</tr>
<tr>
<td>effects of retinoic acid</td>
<td>+/- retinoic acid</td>
<td>↓ SP-A, SP-C mRNA, ↑SP-B mRNA</td>
<td>(22)</td>
</tr>
<tr>
<td>effects of glucocorticoids</td>
<td>+/- dexamethasone</td>
<td>↑ SP-A mRNA at lower concentration but ↓ at concentration &gt; 10&lt;sup&gt;-8&lt;/sup&gt;M</td>
<td>(21)</td>
</tr>
<tr>
<td><strong>VEGF pathway</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>effects of O&lt;sub&gt;2&lt;/sub&gt;, cAMP</td>
<td>2% vs. 20% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↑ VEGF mRNA and protein after 2-4 d in 2% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>(15)</td>
</tr>
<tr>
<td></td>
<td>+/- cAMP</td>
<td>cAMP ↑ VEGF mRNA in 20% but not 2%</td>
<td></td>
</tr>
<tr>
<td>effect of exogenous VEGF</td>
<td>+/- recombinant VEGF</td>
<td>VEGFR-2 expressed in distal airway epithelium</td>
<td>(26)</td>
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<tr>
<td></td>
<td></td>
<td>Exogenous VEGF ↑ epithelium volume density, tissue differentiation and</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ SP-A, SP-C mRNA, SP-B unchanged</td>
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REFERENCES


chapter 6

Cell Proliferation and Apoptosis in Lungs of Patients with Congenital Diaphragmatic Hernia

Prapapan Rajatapiti, Richard Keijzer, Ronald R. de Krijger, Dick Tibboel, and Robbert Rottier
INTRODUCTION

Newborns with congenital diaphragmatic hernia (CDH) may suffer from severe pulmonary hypoplasia and persistent pulmonary hypertension. Despite innovations in prenatal and postnatal care, mortality and morbidity in these patients remain high. The developing fetal lung undergoes dramatic tissue growth and remodeling to achieve the mature structure required for its postnatal role as an air-exchanging organ. Organogenesis of the lung is highly dependent on epithelial-mesenchymal interactions. The fetal lung remains densely cellular after completion of bronchial branching during the pseudoglandular stage. An effective alveolar-capillary interface is then gradually established by regression of the mesenchyme, with simultaneous flattening of the epithelium and outgrowth of capillaries. This process begins in utero during the canalicular and saccular stages of lung development and is completed postnatally during the alveolar stage.

Normal organogenesis requires a delicate balance between cell proliferation, cell differentiation, and cell death. Apoptosis, a form of programmed cell death, has been shown to be involved in several processes during embryogenesis, including involution of web space in limb bud development and kidney and heart development. Several regulatory genes affecting apoptosis have been identified, the most prominent being the Bcl-2 family. Apoptosis is initiated by translocation of pro-apoptotic Bcl-2 family members (e.g. Bax) and is prevented by overexpression of anti-apoptotic molecules such as Bcl-2 or Bcl-XL. In view of the well-established role of apoptosis in embryonic and fetal modeling processes, we hypothesized that apoptosis may play a role in normal and abnormal lung development. Previous studies have suggested that apoptosis is involved in prenatal and postnatal lung remodeling as well. Pulmonary hypoplasia in CDH may be the result of disturbances in normal mechanisms of pulmonary organogenesis such as apoptosis and proliferation. In this study, therefore, we examined the expressions of proteins associated with activation (Bax) or inhibition (Bcl-2) of apoptosis in human CDH lungs. In parallel, we examined these lungs for cell proliferation by immunohistochemical identification of Ki-67 expression.

MATERIALS AND METHODS

Lung tissues

Following approval of the experimental design and protocols by the Erasmus MC Ethical Review Board, lung tissues were retrieved from the tissue bank of the Department of Pathology, Erasmus MC, Rotterdam. We obtained 17 CDH lung samples collected from either termination of pregnancy or patients who died within 48 hours after birth (gestational age 18 - 41 weeks). None of these patients had been subjected to prenatal steroid or extracorporeal membrane oxygenation therapy postnatally. Lung tissue from 20 fetuses and newborns (from termination of pregnancy or autopsies) without...
pulmonary abnormalities served as control material (gestational age 13.5 - 41 weeks). All samples had been harvested within 24 hours after death.

**Immunohistochemistry**

Antibodies used were Ki-67 (clone MIB-1, 1:150, Dako, Heverlee, Belgium), Bax (1:200, Dako, Heverlee, Belgium), and Bcl-2 (clone 124, 1:100, Dako, Heverlee, Belgium). Immunohistochemistry was performed using a ChemMate™ DAKO EnVision™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse (DakoCytomation B.V., Heverlee, Belgium). Immunohistochemical techniques are described in detail in our previous study. Negative controls were performed by omission of the primary antibodies.

**RESULTS AND DISCUSSION**

In this study, we evaluated cell proliferation and expression of apoptotic related proteins during normal human lung development and in human CDH lungs. The proliferation marker Ki-67 was expressed in both airway epithelium and mesenchyme (Figure 6.1). Early in gestation, its expression was more prominent in epithelial cells, with a shift to the mesenchyme, however, later in gestation. Bcl-2 protein expression was detected in the basal layer of airway epithelium and mesenchymal cells in earlier stages of development (figure 6.2 A, B). Later in gestation its expression was also detected in airway epithelial cells (Figure 6.2 C-F). The pro-apoptotic protein Bax was detected in virtually all epithelial cells; moreover it was also detected in pulmonary artery endothelium (Figure 6.3). The pattern of Bax protein expression remained unchanged throughout gestation. There were no striking differences in the expression patterns of Ki-67, Bcl-2, or Bax between normal and CDH lungs. The different levels of the pulmonary vascular tree showed no significant differences between control lungs and CDH lungs. Cell counts were not performed, because postnatal changes could be the result of therapy-related sequelae and postmortem degradation of tissue, making interpretation of the results difficult.

Cell death in multicellular organisms occurs by either necrosis or apoptosis, each of which has distinct morphologic and biochemical characteristics. Cells undergoing apoptosis show shrinkage, nuclear condensation, and DNA fragmentation, but the cytoplasmic membrane remains intact during the early stage. Apoptosis is an active process and proceeds by a multistep cascade involving specific membrane receptors (such as Fas), the Bcl-2 proteins family, and cystein proteases (caspases). The presence of the signaling proteins for apoptosis in the developing lung in our study is not surprising because these proteins are constitutively expressed in many tissues and cell lines. The similar expression of Bcl-2 and Bax between normal and CDH lungs suggests that the disturbance of apoptosis in CDH lungs, if any, occurs largely independent of the Bcl-2/Bax pathway.
Figure 6.1 (for color figures see page 113) Immunohistochemical study of Ki-67 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational ages; 18 weeks (A, B), 23 weeks (C, D), and 35 weeks (E, F). Ki-67 immunoreactivity was detected in both epithelial and mesenchymal cells.
Figure 6.2  (for color figures see page 114) Immunohistochemical study of Bcl-2 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational ages: 18 weeks (A, B), 23 weeks (C, D), and 39 weeks (E, F). Bcl-2, one of the anti-apoptotic proteins, was detected in the basal layer of airway epithelium and mesenchymal cells earlier in development (A, B). Later in gestation its expression was also detected in airway epithelial cells (figure 2 C-F).
Immunohistochemical study of Bax in normal (A, C) and CDH (B, D) lungs at different gestational ages: 23 weeks (A, B), 38 weeks (C, D). Bax immunoreactivity was strongly expressed in cytoplasm of airway epithelial cells. It was also detected in arterial endothelial cells.

**Apoptosis and lung development**

Organ development requires an orchestrated and complex interplay between proliferation, differentiation, and apoptosis. During organ morphogenesis, apoptosis occurs either by direct stimulation or by lack of growth and/or differentiation factors, resulting in the removal of unwanted or excessive cells. Although most of the organogenesis occurs in utero, the lung does not complete its development and maturation until after birth. In humans, alveolarization and microvascular maturation of the lung continues up to at least several years after birth.

Apoptosis was first implicated in the process of lung development some ten years ago. It is observed in mesenchymal cells during the earlier stages of lung development, after which a shift takes place towards both mesenchymal and airway epithelial cells as from the canalicular stage onwards. Throughout embryonic lung development, apoptosis is almost exclusively found in the peripheral mesenchyme – in regions of new bud formation - or in the mesenchyme underlying branch points – the sites of extensive epithelial branching morphogenesis and remodeling of interstitial tissue, allowing room
for outgrowth of the lung bud. Interestingly, mesenchymal cells undergoing apoptosis were intermingled with proliferating mesenchymal cells, suggesting that coordination of these two processes is important in the cell dynamics associated with bronchial branching. One of the mechanisms believed to be involved in regulation of apoptosis during lung development is cellular stretch by mechanical force through liquid secretion or fetal breathing movements.

A delicate balance between apoptosis and proliferation is necessary to support a growing lung and to create room for growth early in gestation. Epithelial apoptosis becomes more important later in gestation with alveolar growth and seems to be stimulated in utero by increasing fetal breathing movements and postnataally by airway distension.

In rabbits, normal fetal lung development was found to be associated with progressively increasing epithelial and mesenchymal apoptotic activity. This process is enhanced by tracheal ligation. Tracheal occlusion (TO) in utero is known to accelerate fetal lung development and was used in selected CDH cases to enhance lung growth and maturation before birth. De Paepe et al. demonstrated that synchronous to the onset of TO-induced distension and alveolar type 2 cell apoptosis, pulmonary FasL protein levels were dramatically higher in TO fetuses, whereas the expressions of Bcl-2 and Bax were similar in control and TO lungs at all time points.

Previous studies in the Nitrofen-induced rat model of CDH suggested a putative role of proliferation and apoptosis in both diaphragm and fetal lung development. While enhanced apoptosis was observed in cervical somites that were considered the precursors of the diaphragm, decreased proliferation was observed in Nitrofen-exposed lungs before diaphragmatic closure. In our studies in human material, no striking differences in cell proliferation between normal and CDH lungs were observed, although no quantitative analysis was performed. We cannot exclude that differences might have become apparent at earlier stages of lung development, for which, however, no human sample was available. Future animal experiments and human data might reveal the relevance of these studies for further insight into the role of proliferation and apoptosis during lung development in CDH cases. So far, diminished proliferation or disturbed balance due to increased apoptosis could not be proven.

Role of apoptosis in development of pulmonary vascular remodeling

Lungs of CDH patients are physically smaller than normal lungs, have fewer airway branches and show vascular abnormalities such as medial hyperplasia of the pulmonary arteries, adventitial thickening, and reduced size of the pulmonary vascular bed. Under normal circumstances, apoptosis has important pathophysiological consequences contributing to the loss of pulmonary smooth muscle cells and therefore in lowering the pulmonary pressure. In recent years, the process of programmed cell death has gained more interest because of its influence on many pathological states. Increased proliferation and decreased apoptosis of pulmonary artery smooth muscle cells could
concurrently mediate thickening of the pulmonary vasculature, which subsequently reduces the luminal diameter of pulmonary arteries, increases pulmonary vascular resistance, and raises pulmonary arterial pressure.\textsuperscript{28,29,31} The balance between apoptosis and cell proliferation is vital for cellular homeostasis. Little is known, however, about the mechanism that coordinates these two processes, particularly in the vessel wall. The role of apoptosis in the pathogenesis and treatment of pulmonary hypertension was recently reviewed by Gurbanov and Shiliang.\textsuperscript{30} They concluded that smooth muscle cells apoptosis is a major feature of blood vessels remodeling and could be a target for new therapeutic approaches for patients with pulmonary hypertension.

With regard to the pulmonary vascular abnormalities in CDH, the medial hyperplasia cannot be explained as the result of a disturbance between proliferation and apoptosis. Ongoing investigations are directed at unraveling the question whether persistence of subpopulations of pulmonary arterial smooth muscle cells contributes to this phenomenon.

In conclusion, modulation of protein expression in the apoptotic cascade and evidence of ongoing cell proliferation during gestation suggest that the lung develops through a coordinated process of cell proliferation coupled with programmed cell death for structural remodeling. Recently our group indeed showed significant changes in matrix metalloproteinases and inhibitors thereof in CDH lungs and control lungs.\textsuperscript{32} While the role of apoptosis in many human diseases has been widely studied, little is known about altered patterns of apoptosis in numerous biologic and clinical fields, including pediatric surgery. The role of apoptosis and proliferation in pulmonary epithelial and vascular remodeling needs further investigation to define the mechanism involved in programmed cell death.
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General Discussion
INTRODUCTION

Congenital diaphragmatic hernia (CDH) has an incidence of 1 in 2500 live births and accounts for approximately 8% of major congenital anomalies. Effective treatment remains one of the major challenges for obstetricians, neonatologists, and pediatric surgeons for various reasons: the diversity of the clinical presentation, the amount of respiratory insufficiency, and the variability of pulmonary hypertension. The past two decades have seen rising numbers of cases diagnosed antenatally. At the same time, greater understanding of the pathophysiology of this condition was reached from insights gained in animal models of CDH and observational clinical studies. Several types of interventions in utero have been proposed to address CDH, but these efforts have met with variable success and are currently applied in very few institutions under experimental protocols for which informed consent is warranted. Despite improved clinical care of infants with CDH, the overall mortality rate for live born infants remains above 30% and is even higher if all prenatally identified fetuses are included. Importantly, there is also significant morbidity among the CDH survivors, including developmental delay, gastroesophageal reflux, growth failure, and chronic pulmonary disease. This group of patients therefore requires long-term follow up.

The unpredictable response to postnatal treatment has led to the idea that correcting the defect and/or promoting lung growth prenatally might increase survival and bring down morbidity of infants with CDH – and thus would be the ultimate therapeutic goal. This strategy nevertheless requires better understanding of the mechanism(s) controlling lung development in CDH. Most current therapeutic recommendations are based primarily on observational studies and integration from findings of animal studies in clinical practice. The results from animal studies need to be further evaluated, however, before application for clinical use in CDH patients is feasible. This calls for setting up properly designed trials with enough power.

PHARMACOLOGICAL MODULATION OF LUNG GROWTH

As the benefit of fetal surgical intervention in utero remains unclear, pharmacological approaches to stimulate prenatal lung growth and maturation have been considered as an alternative strategy to improve the outcome in children with CDH. Several hormones studied in a variety of animal studies are suggested to affect lung maturation. Glucocorticoids and thyroid hormones seem to be the most important candidates for clinical practice. Morphological similarities between the lung of premature newborns and infants with CDH have led to the hypothesis that antenatal glucocorticoids (GC) might have beneficial roles. Moreover, changes in thyroid hormone metabolism are thought to be involved in the pathogenesis of Nitrofen-induced CDH in rats. Prenatal administration of GC resulted in improved pulmonary compliance, narrowed septal walls, and thinning of pulmonary interstitium in rats with Nitrofen-induced CDH and a combination of GC and thyrotropin releasing hormone enhanced these effects.
Recently, more attention has focused on vitamin A and its active form, retinoic acid (RA). Vitamin A plays an important role in lung development. The first evidence linking retinoids with CDH came from the observation of diaphragmatic defects in pups born to vitamin A-deficient dams as early as the mid 1940s. Studies of retinoid receptor double null-mutant mice, lacking both the α and β subunits of retinoic acid receptors (RARs), have demonstrated that some offspring have a diaphragmatic defect similar to that observed in human CDH. Moreover, co-administration of vitamin A and Nitrofen reduces the incidence of CDH in rats by 15 to 30%. These experimental observations suggest that abnormalities within the retinoid signaling pathway may contribute to the etiology of CDH, at least in rodent models. To date only one study suggests a relationship between RA and CDH in humans. Importantly, enzymes involved in degradation of RA, such as RALDH-2, are localized on chromosome 15.

The actions of glucocorticoids, thyroid hormone, and retinoic acid are mediated through their specific nuclear receptors and the associated expression levels of the receptors form indications for the cellular responsiveness towards certain ligands. For this reason we examined the expression of these receptors during human lung development (chapter 2). Expressions of the glucocorticoid receptor (GR) α, thyroid hormone receptor (TR) α and β, retinoic acid receptors (RARs), and retinoid X receptors (RXRs) were detected as early as the pseudoglandular stage of lung development. GRα was ubiquitously expressed in both epithelium and mesenchyme, albeit more pronounced in epithelium. While TRα was expressed in both epithelium and mesenchyme, TRβ was detected predominantly in the pulmonary epithelium. Furthermore, the expression patterns of these receptors were evaluated in lungs of CDH patients and hypoplastic lungs resulting from other causes (chapter 3). No differences in timing and localization of these receptors were observed between normal and hypoplastic lungs. Comparable findings were demonstrated in lungs of control and Nitrofen-induced CDH rats. Based on the information from experimental studies, which demonstrated the potential benefit of antenatal GC and vitamin A to promote lung growth and maturation in an animal model of CDH, our results indicate that the human CDH lung as well has the potential for a similar response to pharmacological modulation. A planned GC study in human prenatal CDH cases failed completely because it appeared not possible to include patients in and many centers were refused Institutional Board Review approval.

As recommended by the American National Institutes of Health, the use of antenatal GC for CDH was adopted for all fetuses between 24 to 34 weeks of gestation at risk of preterm delivery. However, a caution was raised about the use of this therapy in CDH, in view of the paucity of human data, the risks of repetitive antenatal steroid administration, and lack of information on dosage and timing. Recent data from a randomized trial and cohort study performed by the CDH Study Group showed no significant benefit of late prenatal GC in fetuses with CDH and therefore its use is not recommended beyond 34 weeks of pregnancy. Current interest has turned towards retinoic acid since there is strong evidence that the retinoid signaling pathway is involved...
in the Nitrofen-induced CDH model. Such evidence is still lacking in human CDH and it remains unclear whether retinoids can reduce the severity of pulmonary hypoplasia. Aimed at elucidating the potential link between retinoids and human CDH, an international multicenter study on retinoids level in plasma from infants with CDH and their mothers is ongoing. Further investigations are required in order to assess the real potential and/or possible teratogenic risk of vitamin A during the prenatal period.

Moreover, identification of low vitamin A status in mothers who will deliver a child with CDH is hampered by the low incidence of CDH (1:3000). While major population based studies might solve this problem, we could be confronted with a wide variability between countries and populations.

**PULMONARY VASCULATURE AND AIRWAY BRANCHING MORPHOGENESIS**

From the first morphological sign of lung development, a vascular network surrounds the emerging lung buds and expands by formation of new capillaries from preexisting vessels as the lung buds grow into the surrounding mesenchyme. This angiogenesis process is mainly driven by hypoxia. Vascular endothelial growth factor (VEGF) appears to be one of the most potent hypoxia-dependent angiogenic growth factors especially during lung development. VEGF signaling is mediated via its receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), and VEGFR-3 (Flt-4). Expression of VEGFR-1 and VEGFR-2 is principally in the vascular endothelium, whereas VEGFR-3 is mainly expressed in lymphatic endothelium. Among these three receptors, VEGFR-2 plays an important role in angiogenesis and is one of the earliest molecular indicators of the endothelial cell precursor.

VEGF mRNA expression is regulated through hypoxia inducible factor (HIF), which in turn is controlled at the protein level by von Hippel-Lindau protein (VHL).

In the human lung, VEGF and its receptors are localized primarily in airway epithelium and vascular endothelium respectively. Epithelial-endothelial interactions during lung development are essential for the establishment of a functional blood-gas interface. Localization of the VEGF protein in the basement membrane of airway epithelium may be important for directing capillary development in the human lung through its paracrine effects on endothelial cells. Various studies have provided evidence that the developing pulmonary vasculature has an important regulatory role in airway branching morphogenesis. Precise spatial expression and temporal activation of VEGF is required for vascular patterning during lung morphogenesis. Fetal lambs showed a three-fold increase in VEGF expression before birth, despite a strong expression of VEGF protein in the lung during early and mid-gestation. Our study in human lungs of different developmental stages demonstrated comparable expression patterns of VEGF mRNA during development. Moreover, mRNA expression of HIF-2α, a potent activator of VEGF, also increased with advancing gestation. Our data support the role of the VEGF signaling cascade in primitive vascular network formation as well as in preparing the fetal lung for the extra uterine environment in the final phase of gestation.
There is evidence for morphological abnormalities of the lung vasculature in CDH patients. Hypoplastic lungs with abnormal, thick-walled pulmonary arteries and a contracted pulmonary microvascular bed may cause pulmonary hypertension in the newborn. Similar abnormalities in the pulmonary vessels as in CDH patients have been observed in the Nitrofen-induced CDH rat model. Immunohistochemistry revealed lower VEGF expression in lungs of CDH rats. However, contrasting results have been reported for the human CDH lungs. These were found to show altered expression of other angiogenesis-related factors – i.e. increased expression of VHL and decreased expression of HIF-1α – compared with age-match control lungs. Despite some controversial results, these data suggest a role for the VEGF signaling pathway in normal and abnormal pulmonary angiogenesis, especially in CDH patients. To date no experimental approaches to alter VEGF expression in models of CDH have been published as precursors for human studies. Some clinics apply inspiration of the mother with 100% oxygen to predict the postnatal vascular reactivity of the pulmonary circulation. So far the results are inconclusive, as more subjects are needed to evaluate the relative value of these observations.

Prenatal evaluation of echo Doppler flow pattern of the contralateral lung during prenatal ultrasound has never resulted in values predicting postnatal vasodilatory capacity of the pulmonary vasculature.

**ROLE OF OXYGEN IN PULMONARY DEVELOPMENT**

Normal pulmonary development takes place in the relatively hypoxic environment in utero. Hypoxia is one of the most important factors that drive pulmonary development in the embryo. Hypoxia-induced VEGF expression is mediated by molecular oxygen sensors, the hypoxia-inducible factors (HIF). Van Tuyl et al. demonstrated that a relatively hypoxic environment (3% oxygen) stimulates pulmonary vasculature and epithelial branching morphogenesis at least in mouse lungs in vitro. In this thesis (chapter 5), we demonstrate that a low oxygen environment (1.5% oxygen) stimulates VEGF mRNA expression in human lung explants in vitro; no significant changes in mRNA expressions of VEGFR-2, HIF-2α, and HIF-3α were observed. We must be aware, however, that HIF expression is mainly regulated at the posttranscriptional level, and hence further investigation at the protein level is needed for better understanding of the role of HIFs in the lung under hypoxic conditions. The use of 100% oxygen during artificial ventilation postnatally may have a serious impact on expression of ‘hypoxia’ dependent transcription factors and a potentially negative effect on pulmonary vascular growth.

Although upregulation of potent angiogenic factors such as VEGF has been demonstrated in hypoxic lung cultures, more research is required to prove that an increase in VEGF (indirectly) induces airway branching morphogenesis. Extrinsic factors such as a hypoxic environment may use the VEGF signaling pathway either directly or
through other mediators, to produce an angiogenic effect, which in turn promotes pulmonary vascular development. It will not be easy to identify the key molecule in this highly orchestrated process during pulmonary development, which is not only regulated by VEGF.

This knowledge could be crucial in the care of premature babies. Their lungs normally should have developed for a longer period in the relatively hypoxic environment of the uterus. Hyperoxic exposure dramatically decreases lung VEGF mRNA expression. Blocking of VEGF or inhibition of its receptor, VEGFR-2, during fetal lung development results in inhibition of angiogenesis and alveolar hypoplasia, producing pathologic features resembling bronchopulmonary dysplasia. The effect of hyperoxia on postnatal pulmonary development in CDH cannot easily be investigated. In clinical conditions high inspiratory oxygen fractions are needed with a potential negative effect on the pulmonary vasculature. The high incidence of BPD in term born CDH patients might reflect this negative effect on the pulmonary vasculature.

**CONCLUDING REMARKS**

Although extensive research efforts have attempted to elucidate the etiology of CDH and associated pulmonary hypertension, many issues remain to be clarified. The use of different animal models and materials from human CDH patients revealed confusing and sometimes contradicting data. We need to interpret these results and implicate these in our clinical practice. Full understanding of the pathophysiology of this disease and of the factors that contribute to lung injury will eventually guide the prenatal, perinatal, and postnatal management of patients with CDH. Most of the current treatment strategies are based on data from animal studies. However, treatment modalities should be further studied in properly designed randomized controlled trials, if feasible. International collaboration of the CDH Study Group provides important data in this respect, for example on the use of prenatal corticosteroids after prenatal diagnosis. Beneficial effects of pharmacological modulation, especially vitamin A, have been reported in the Nitrofen-induced rat model but the exact mechanisms behind these effects remain unknown. The large number of suggested interventions demonstrates that we still have not found the optimal solution to improve lung growth or to manage the hypoplastic lung and pulmonary hypertension in CDH.
REFERENCES


Chapter 8

SUMMARY

Congenital diaphragmatic hernia (CDH) is a congenital anomaly with an incidence of one in 2,000 to 3,000 live births. The pathophysiology is a mixture of pulmonary vascular and pulmonary alveolar abnormalities. Pulmonary hypoplasia and persistent pulmonary hypertension are the primary causes of morbidity and mortality in infants with congenital diaphragmatic hernia. An extensive volume of work has been performed by a multitude of investigators in an attempt to explain the origins of CDH and associated pulmonary hyperplasia and hypertension. Nevertheless, to date our understanding of the pathophysiology of this disease is still incomplete. However, these studies have yielded important insights into mediators and mechanisms of normal lung and diaphragmatic growth and development. In addition, some of these investigations have suggested new opportunities for intervention. Apart from advances in postnatal management, there have been attempts at prenatal intervention aimed at either repairing the defect or enhancing lung growth prior to birth.

Chapter 1 describes clinical features of CDH and discusses the variety of current treatment strategies as well as the possibilities of prenatal modulations to promote lung growth and maturation. Finally it presents the aims of the reported studies.

Chapter 2 describes expression patterns of nuclear hormone receptors in a series of normal human fetal lungs from 13.5 weeks of gestation till term. Glucocorticoids, thyroid hormone, and vitamin A have been described to influence prenatal lung growth. The actions of these ligands are mediated through activation of their receptors, and the level of nuclear receptor expression determines cell sensitivities to certain hormones. The presence of these receptors in human lung has been documented; however, the ontogeny of their expressions has not been shown systematically. The expressions of glucocorticoid receptor (GR) α, thyroid hormone receptor (TR) α and β, retinoic acid receptor (RAR) α, β, γ, and retinoid X receptor (RXR) α, β, γ were evaluated by quantitative PCR and immunohistochemical studies.

As early as 13.5 week of gestation or pseudoglandular stage of lung development, mRNA and protein expression of all receptors were detected in human lungs. These findings indicate that, as far as receptors are concerned, human fetal lung has the potential to respond to glucocorticoids, thyroid hormone, and retinoic acid as early as in the pseudoglandular stage, which implies a potential for therapeutic or toxic effects by exogenous ligands.

These results in normal human lungs were used as baseline data for the study in chapter 3 in which expression patterns of these receptors were evaluated in lungs of human CDH cases. The mRNA expression of these receptors was analyzed in 5 CDH cases, and immunohistochemistry was performed in 18 CDH cases and 20 cases of pulmonary hypoplasia from other causes. As a series of supportive experiments, we
examined expression patterns of these receptors in lungs of controls and Nitrofen-induced CDH rats.

Immunohistochemistry (human and rat) and in situ hybridization (rat) demonstrated no overt difference between CDH, hypoplastic, and control lungs, neither in the localization nor the timing of the first expression of all analyzed receptors. The mRNA expression of each receptor was detected in all human CDH lungs by quantitative PCR. These results suggest that the hypoplastic lungs of fetuses or newborns with CDH are potentially as responsive to glucocorticoids, thyroid hormone, and retinoic acid as the lungs of normal children. Recently, vitamin A has been shown to lower the incidence of CDH in the Nitrofen-induced CDH rat model; our results imply a possibility of similar benefit in human CDH cases.

In chapter 4 the expressions of various angiogenesis-related factors were evaluated in a series of normal human lungs from 13.5 weeks of gestation onwards till term. Hypoxia-inducible factors (HIF) appear to be key molecules for angiogenesis. However, documentation of qualitative and quantitative expression of HiFs in normal human fetal lung development was limited. For this reason we evaluated expressions of HIF-1α, HIF-2α, and HIF-3α along with von Hippel-Lindau protein (VHL), vascular endothelial growth factor A (VEGF-A) and its receptor, VEGFR-2 by quantitative PCR. Both HIF-2α and VEGF-A mRNA expression showed increasing trend during gestation. Furthermore, localization of HIF-1α and HIF-2α was evaluated by immunohistochemistry and this revealed that while HIF-1α is expressed in epithelial cells only, HIF-2α is expressed in both epithelial and mesenchymal cells.

These findings support the role of the VHL-HIF-VEGF pathway in pulmonary vascular development and airway branching morphogenesis in the human fetal lung.

Chapter 5 describes the effects of low oxygen environment on expressions of HIFs in human lung in vitro. Human lung explants cultured at 1.5% oxygen were used to replicate physiologic conditions during normal fetal lung development. VEGF-A mRNA expression is significantly upregulated in explants cultures at 1.5% oxygen. Although slight increases in HIF-2α and HIF-3α mRNA expression were observed, the differences are not significant. These data suggest that the expression of VEGF-A – and possibly HIF-2α and HIF-3α – is regulated by hypoxia in the developing human lung. Moreover the experiments in this chapter demonstrate that human fetal lung culture is a valuable model to unravel the mechanisms of factors that are known to modulate fetal lung development, such as retinoic acid and hormones.

In chapter 6 we investigate cell proliferation and apoptotic related protein in series of normal fetal lungs and human CDH lungs during development. Proliferating cells were observed in both epithelial and mesenchymal cells. Normal organogenesis requires an optimal balance between cell proliferation, cell differentiation, and cell death. The role of
apoptosis in pulmonary airway and vascular development was recently described. However, further investigation is needed to elucidate the mechanisms involved in programmed cell death in pulmonary epithelial and vascular remodeling; we may then gain better understanding of the pathophysiology of abnormal lung development as in cases of CDH.

Chapter 7 provides a general discussion on pharmacological modulations of fetal lung growth, pulmonary vasculature and airway branching morphogenesis; and the role of oxygen on pulmonary development.

In conclusion, CDH remains a major challenge to neonatologists and pediatric surgeons. In view of the vast variety of clinical features, treatment strategies should be adapted to the individual patient. Although many treatment modalities are used in CDH, most of them are based on information from animal experiments. The obstacles to obtain human data are due to the relatively low incidence of CDH and lack of good international collaboration. Only by establishing networks of centers in which enough infants with CDH are managed will we be able to conduct appropriately sized randomized trials that can contribute to solving some of the crucial dilemmas of the management of these infants and can shed light onto their long-term outcome.
Congenitale hernia diafragmatica (CHD) is een aangeboren afwijking die voorkomt bij één op de 2000 tot 3000 pasgeboren. De fysieke uitingen hiervan vormen een combinatie van aandoeningen aan de longvaten en de longalveolen. Longhypoplasie en persistierende pulmonale hypertensie zijn de belangrijkste oorzaken van de morbiditeit en mortaliteit bij kinderen met deze afwijking. Alhoewel er al veel energie en menskracht is gestoken in het zoeken naar de oorsprong van CHD en de samengaande longhyperplasie en -hypertensie, is het beeld dat wij ervan hebben nog steeds incompleet. Niettemin hebben al die onderzoeken waardevol inzicht opgeleverd in de mediators en mechanismen van de normale groei en ontwikkeling van de longen en het middenrif. Tevens zijn er suggesties voor nieuwe interventiemogelijkheden uit voortgekomen. Er is niet alleen vooruitgang geboekt in de aanpak vlak na de geboorte; ook is er gewerkt aan prenatale interventie, enerzijds gericht op herstel van het middenrifdefect, anderzijds op het bevorderen van de longgroei voor de geboorte.

Hoofdstuk 1 beschrijft de klinische kenmerken van CHD en gaat in op de diverse beschikbare behandelingmethoden en de mogelijkheden van prenatale modulatie om de groei en rijping van de longen te bevorderen. Het hoofdstuk sluit af met de doelstellingen van de onderzoeken die in dit proefschrift worden gepresenteerd.

Hoofdstuk 2 beschrijft de expressiepatronen van nucleaire hormoonreceptoren in een reeks normale longpreparaten van menselijke foetussen vanaf 13,5 weken tot voldragen zwangerschap. Van glucocorticoïden, schildklierhormoon en vitamine A is aangetoond dat ze de prenatale longgroei beïnvloeden. Deze bindingseiwitten komen in werking als hun receptoren worden geactiveerd, en het expressieniveau van de nucleaire receptoren bepaalt in hoeverre cellen gevoelig zijn voor bepaalde hormonen. Er is aangetoond dat deze receptoren voorkomen in de longen van de mens; de ontwikkeling van hun expressies is echter nog niet systematisch onderzocht. De expressies van de glucocorticoidreceptor (GR) α, schildklierhormoonreceptor (TR) α en β, de retinoïnezuurreceptor (RAR) α, β, γ, en retinoïnezuurreceptor-X (RXR) α, β, γ werden geëvalueerd met behulp van kwantitatieve PCR en immunohistochemisch onderzoek.

Al bij 13,5 week zwangerschap, of de pseudoglandulaire fase van de longontwikkeling, vonden we mRNA- en eiwitexpressie van alle receptoren in deze longpreparaten van menselijk foetussen. Onze bevindingen wijzen er op dat – wat betreft de receptoren – de longen van ongeboren kinderen al in de pseudoglandulaire fase in staat zijn te reageren op glucocorticoiden, schildklierhormoon en retinoïnezuurreceptor. Dit geeft aan dat exogene bindingseiwitten wellicht therapeutisch of toxisch effect kunnen hebben.

Wat we hadden gevonden voor de normale longontwikkeling bij de mens diende als basisgegevens voor het onderzoek dat in Hoofdstuk 3 is beschreven. Nu werden de expressiepatronen van deze receptoren bekeken in longmateriaal van kinderen met CHD. In vijf gevallen werd de mRNA-expressie van deze receptoren geanalyseerd, en in
18 gevallen werd immunohistochemisch onderzoek gedaan; dit laatste werd tevens gedaan voor 20 gevallen van longhypoplasie door andere oorzaken. Bij wijze van ondersteunend onderzoek hebben we ook de expressiepatronen van deze receptoren onderzocht bij kinderen uit een controlegroep en bij ratten met CHD geïnduceerd door Nitrofen.

Immunohistochemisch onderzoek (mens en rat) en in situ hybridisatie (rat) toonden voor alle geanalyseerde receptoren geen opmerkelijke verschillen tussen de longen in geval van CHD, van hypoplasie, en van de controlegroep, noch in de lokalisatie, noch in het tijdstip waarop de expressie het eerst zichtbaar was. De mRNA-expressie van elke receptor werd met behulp van kwantitatieve PCR aangetoond in alle longmateriaal van kinderen met CHD. Deze resultaten geven aan dat de hypoplastische longen van foetussen of pasgeboren met CHD potenteel net zo kunnen reageren op glucocorticoiden, schildklierhormoon en retinoïnezuurreceptor als de longen van normale kinderen. Onlangs heeft men aangetoond dat vitamine A leidt tot lagere incidentie van CHD in het Nitrofen-rattenmodel; onze bevindingen impliceren dat een zelfde gunstig resultaat kan worden bereikt bij kinderen met CHD.

In Hoofdstuk 4 werd de expressie onderzocht van diverse angiogenese-gerelateerde factoren in een serie normale menselijke longen vanaf 13,5 week tot aan het eind van de zwangerschap. Hypoxie-induceerbare factors (HIF’s) bleken de sleutelmoleculen te zijn voor de angiogenese. De kwalitatieve en kwantitatieve expressie van HIF’s in de normale longontwikkeling bij menselijke foetussen is echter nog maar beperkt gedocumenteerd. Daarom hebben we met behulp van kwantitatieve PCR de expressie van HIF-1α, HIF-2α, en HIF-3α geëvalueerd, alsmede die van Von Hippel-Lindau proteïne (VHL), vasculaire endotheel groeifactoren A (VEGF-A) en de receptor ervan, VEGFR-2. Zowel de expressie van HIF-2α als die van VEGF-A mRNA vertoonden een stijgende trend over de zwangerschap. Vervolgens keken we met behulp van immunohistochemisch onderzoek naar de lokalisatie van HIF-1α en HIF-2α. Het bleek dat HIF-1α alleen maar in epitheelcellen tot uitdrukking kwam, terwijl HIF-2α zowel in epitheelcellen als in mesenchymale cellen te zien was.

Deze bevindingen ondersteunen de theorie dat de VHL-HIF-VEGF pathway een rol speelt bij de ontwikkeling van de longvaten en bij de morfogenese van de vertakking van de luchtwegen in de menselijk foetale long.

Hoofdstuk 5 beschrijft de effecten van laag zuurstofgehalte van de omgeving op de expressie van HIF’s in de menselijke long in vitro. Explantaten van menselijke longen gekweekt in 1,5% zuurstof dienden om de fysiologische omstandigheden tijdens normale foetale longontwikkeling te repliceren. De VEGF-A mRNA-expressie was significant verhoogd in deze explantaten. Lichte verhogingen werden waargenomen in de expressie van HIF-2α en HIF-3α mRNA, maar de verschillen zijn niet significant. Deze bevindingen suggereren dat de expressie van VEGF-A – en mogelijk HIF-2α and HIF-3α – wordt
gereguleerd door hypoxie in de zich ontwikkelende menselijk long. Bovendien tonen de experimenten in dit hoofdstuk aan dat longkweek van menselijke foetussen een waardevol model vormt voor het ontrafelen van de werkingsmechanismen van factoren waarvan we weten dat ze de foetale longontwikkeling moduleren, zoals retinoïnezuur receptor en hormonen.

In Hoofdstuk 6 onderzochten we de celproliferatie and apoptose-gerelateerde proteïne in series longpreparaten van normale foetussen en van kinderen met CHD tijdens de ontwikkeling. We zagen proliferatie van zowel epitheelcellen als mesenchymale cellen. De normale organogenese is gestoeld op een optimale balans tussen celproliferatie, celdifferentie, en cel dood. De rol van apoptose bij de ontwikkeling van de luchtwegen en bloedvaten in de longen werd onlangs nog beschreven. Er is echter nader onderzoek nodig om de mechanismen te achterhalen die betrokken zijn bij de geprogrammeerde cel dood die zich voordoet bij de remodeling van longepitheel en -vaten; dit zou ons dan een beter begrip geven van de fysieke uitingen van abnormale longontwikkeling zoals in gevallen van CHD.

Hoofdstuk 7 brengt een algemene discussie betreffende farmacologische modulering van foetale longgroei, het vaatstelsel en de morfogenese van de vertakking van de luchtwegen in de longen, en de rol van zuurstof bij de longontwikkeling.

De conclusie luidt dat congenitale hernia diafragmatica nog steeds een belangrijke uitdaging vormt voor neonatologen en kinderchirurgen. Aangezien de klinische kenmerken sterk uiteenlopen is het zaak om de te volgen behandelingstrategie aan te passen aan de individuele patiënt. Er zijn vele methoden om de behandeling van CHD aan te pakken, maar de meeste daarvan zijn gebaseerd op informatie verkregen uit dierexperimenten. Gegevensverzameling uit onderzoek bij kinderen wordt belemmerd door de relatief geringe incidentie van CHD en het gebrek aan goede internationale samenwerking. Alleen als we netwerken vormen van medische centra met voldoende aantallen kinderen met CHD kunnen we gerandomiseerde trials van voldoende omvang opzetten. Die kunnen dan bijdragen aan het zoeken naar een oplossing voor bepaalde cruciale dilemma’s bij de behandeling van die kinderen, en kunnen licht werpen op wat deze kinderen op de lange termijn te wachten staat.
Curriculum Vitae
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WORDS OF APPRECIATION

When I decided to come to the Netherlands to pursue my PhD, I had no idea what was lying ahead of me. I am most grateful to Professor (or in Thai I call him ‘Ajarn’) Soottiporn Chittmittrapap for his kindness and support which encouraged me to pursue advanced research for the benefit of our institution in the future. I would like to acknowledge the Faculty of Medicine, Chulalongkorn University, for the generous scholarship that has made my PhD study in Erasmus MC possible.

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Finally, I would like to thank my family and friends in Thailand whose support has been indispensable. Especially my father, who showed me how to be a good doctor and a good teacher. I hope one day I can be at least half as good as him.

Prapapan
Color Figures
Figure 2.3  (see page 20) Immunostaining of representative samples of human fetal lung. All pictures were taken from sample of 18 weeks of gestation except for TRβ (32 wk) and RXRβ (13.5 wk). The immunoreactivity was visualized by diaminobenzidine, positive cells giving a brown color at the site of reaction. (A), GR immunoreactivity was expressed mainly in nuclei of epithelial cells. (B), TRα was detected in both epithelial and mesenchymal cells, while TRβ (C), was detected in airway epithelium and some vascular endothelial cells (arrow). Immunoreactivities of RARα (D), RARβ (E), RARγ (F), RXRα (G), RXRβ (H), and RXRγ (I) were detected virtually in all epithelial and in some of mesenchymal cells. (scale bar, 50 μm)
Figure 3.2 (see page 35) TRα and RXRγ expression pattern in human and rat lungs. TRα (A-E) and RXRγ (F-J) are seen in both epithelial and mesenchymal cells. There is no difference in the localization of TRα or RXRγ in the lungs of human control; 23 wk (A, F), CDH: 34 wk (B, G), or pulmonary hypoplasia due to other causes: 27 wk (C, H). Comparable patterns are observed in control (D, I) and nitrogen-induced CDH (E, J) rat lungs (d 18 of gestation). All pictures were taken at the same magnification; scale bar = 100 μm.
Figure 4.3 (see page 50) HIF-1α immunostaining with strong expression in airway epithelium of human fetal lung at different gestational ages: (a) 16 wk, (b) 21 wk, (c) 27 wk, and (d) 31 wk. (Magnification, X 400)
Figure 4.4 (see page 50) HIF-2α immunostaining with predominant reactivity in the interstitium at early stages of human fetal lung development; (a) 16 wk, (b) 21 wk. Later on in gestation, HIF-2α expression was also detected in type II pneumocytes; (c) 27 wk, and (d) 33 wk. (Magnification, X 400)
Figure 5.1  (see page 62) Morphology of human fetal lung explants cultured at 20% oxygen. Hematoxylin & Eosin staining of uncultured lung (A) resembled the pseudoglandular stage of lung development. The airways were dilated after the explants were cultured for 3 (B) and 6 days (C) at 20% oxygen. Bar, 500 μm.
Figure 5.2  (see page 63) Immunohistochemical staining with TTF-1, CD-31, and Ki-67 of uncultured lung (A-C), explants cultured at 20% for 3 (D-F) or 6 days (G-I), and 1.5% oxygen for 6 days (J-L). TTF-1 immunoreactivity is detected in epithelial cells (A, D, G, J). Explants cultured at 1.5% oxygen (K) showed stronger CD-31 staining in the endothelial cells compared with control (B) and explants cultured at 20% oxygen (E, H). Ki-67 staining (C, F, I, L) showed proliferating epithelial cells and mesenchymal cells in control (C) and explants kept at 20% oxygen (F, I), whereas the expression is more restricted to mesenchyme in explants kept at 1.5% oxygen (L). Bar, 500 μm.
Figure 5.3  (see page 64) Double immunostaining of Ki-67 and CD-31 on explants cultured at 20% oxygen for 6 days. Ki-67 (A) immunoreactivity is detected in epithelium and mesenchyme. Double staining with CD-31 (pink staining) shows proliferating endothelial cells (arrows; B). Bar, 250 μm.
Figure 6.1  (see page 76) Immunohistochemical study of Ki-67 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational ages; 18 weeks (A, B), 23 weeks (C, D), and 35 weeks (E, F). Ki-67 immunoreactivity was detected in both epithelial and mesenchymal cells.
Figure 6.2 (see page 77) Immunohistochemical study of Bcl-2 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational ages: 18 weeks (A, B), 23 weeks (C, D), and 39 weeks (E, F). Bcl-2, one of the anti-apoptotic proteins, was detected in the basal layer of airway epithelium and mesenchymal cells earlier in development (A, B). Later in gestation its expression was also detected in airway epithelial cells (figure 2 C-F).
Figure 6.3 (see page 78) Immunohistochemical study of Bax in normal (A, C) and CDH (B, D) lungs at different gestational ages; 23 weeks (A, B), 38 weeks (C, D). Bax immunoreactivity was strongly expressed in cytoplasm of airway epithelial cells. It was also detected in arterial endothelial cells.
Figure 2.3 (see page 20) Immunostaining of representative samples of human fetal lung. All pictures were taken from samples of 18 weeks of gestation except for TRβ (32 wk) and RXRβ (13.5 wk). The immunoreactivity was visualized by diaminobenzidine, positive cells giving a brown color at the site of reaction. (A), GR immunoreactivity was expressed mainly in nuclei of epithelial cells. (B), TRα was detected in both epithelial and mesenchymal cells, while TRβ (C), was detected in airway epithelium and some vascular endothelial cells (arrow). Immunoreactivities of RARα (D), RARβ (E), RARγ (F), RXRα (G), RXRβ (H), and RXRγ (I) were detected virtually in all epithelial and in some of mesenchymal cells. (scale bar, 50 μm)
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Figure 4.4 (see page 50) HIF-2α immunostaining with predominant reactivity in the interstitium at early stages of human fetal lung development: (a) 16 wk, (b) 21 wk. Later on in gestation, HIF-2α expression was also detected in type II pneumocytes; (c) 27 wk, and (d) 33 wk. (Magnification, X 400)
Figure 5.1  (see page 62) Morphology of human fetal lung explants cultured at 20% oxygen. Hematoxylin & Eosin staining of uncultured lung (A) resembled the pseudoglandular stage of lung development. The airways were dilated after the explants were cultured for 3 (B) and 6 days (C) at 20% oxygen. Bar, 500 μm.
**Figure 5.2** (see page 63) Immunohistochemical staining with TTF-1, CD-31, and Ki-67 of uncultured lung (A-C), explants cultured at 20% for 3 (D-F) or 6 days (G-I), and 1.5% oxygen for 6 days (J-L). TTF-1 immunoreactivity is detected in epithelial cells (A, D, G, J). Explants cultured at 1.5% oxygen (K) showed stronger CD-31 staining in the endothelial cells compared with control (B) and explants cultured at 20% oxygen (E, H). Ki-67 staining (C, F, I, L) showed proliferating epithelial cells and mesenchymal cells in control (C) and explants kept at 20% oxygen (F, I), whereas the expression is more restricted to mesenchyme in explants kept at 1.5% oxygen (L). Bar, 500 μm.
Figure 5.3  (see page 64) Double immunostaining of Ki-67 and CD-31 on explants cultured at 20% oxygen for 6 days. Ki-67 (A) immunoreactivity is detected in epithelium and mesenchyme. Double staining with CD-31 (pink staining) shows proliferating endothelial cells (arrows; B). Bar, 250 μm.
Figure 6.1 (see page 76) Immunohistochemical study of Ki-67 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational ages: 18 weeks (A, B), 23 weeks (C, D), and 35 weeks (E, F). Ki-67 immunoreactivity was detected in both epithelial and mesenchymal cells.
Figure 6.2 (see page 77) Immunohistochemical study of Bcl-2 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational ages: 18 weeks (A, B), 23 weeks (C, D), and 39 weeks (E, F). Bcl-2, one of the anti-apoptotic proteins, was detected in the basal layer of airway epithelium and mesenchymal cells earlier in development (A, B). Later in gestation its expression was also detected in airway epithelial cells (figure 2 C-F).
Figure 6.3 (see page 78) Immunohistochemical study of Bax in normal (A, C) and CDH (B, D) lungs at different gestational ages; 23 weeks (A, B), 38 weeks (C, D). Bax immunoreactivity was strongly expressed in cytoplasm of airway epithelial cells. It was also detected in arterial endothelial cells.